

**THE EFFECT OF CONDENSED TANNINS AND DIETARY  
PROTEIN ON RUMINANT INTESTINAL NEMATODE  
INFECTIONS**

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## ABSTRACT

Endoparasitic control is still heavily reliant on the use of anthelmintic drugs, however, frequent use and mis-use of anthelmintics is leading to the development of multiple resistance. In the tropics and subtropics where marginal levels of nutrition lead to greater susceptibility to infection, animal death due to nematode infection remains widely apparent. Here, anthelmintics are either unaffordable, of inferior quality or extensive multiple resistance has made these drugs ineffective. Consequently, alternative methods of parasitic control are required that are practical and realistic for introduction into farm production systems. One such possibility could be the exploitation of forage species capable of reducing parasitic infection solely, or in conjunction, with limited drug use. In tropical and subtropical regions many plants contain condensed tannins due to stress induced by environmental conditions. Sheep fed forages containing condensed tannins have been reported to have reduced gastrointestinal nematode infections. The aim of the studies reported in this thesis was to determine whether the inclusion of a model condensed tannin, quebracho tannin, and/or the elevation of dietary protein could reduce the establishment and persistence of small intestinal nematode infections.

Initial work using the *Nippostrongylus brasiliensis*-rat model demonstrated that the inclusion of 40 g quebracho tannin/kg in both high and low protein diets significantly ( $p < 0.05$ ) reduced the number of *N. brasiliensis* establishing in the small intestine. Mean total daily faecal egg counts were also significantly ( $p < 0.05$ ) reduced by dietary quebracho tannin, and high dietary protein concentration. Data obtained from using the *Trichostrongylus colubriformis*-sheep model demonstrated that faecal egg counts were significantly ( $p < 0.05$ ) reduced when 50 g quebracho tannin/kg was included in a low protein diet. Increasing the dietary protein concentrations also reduced faecal egg counts to similar levels. The inclusion of quebracho tannin in a high protein diet did not significantly ( $p > 0.2$ ) reduce total daily faecal egg counts. Haematological and serological parameters did not show any significant ( $p > 0.2$ ) differences between dietary treatments. Subsequent investigations showed that dietary quebracho tannin was not reducing worm establishment and persistence by elevating the host immune response. Further studies suggested that quebracho tannin was acting through a direct toxic effect against the worm, where the mucosal inhabiting nematode, *Trichinella spiralis*, was unaffected by the presence of dietary quebracho tannin and *in vitro* data where *N. brasiliensis* survival was compromised by incubating worms in quebracho tannin-containing media. Concentrations as low as 0.01% (w/v) quebracho tannin proved effective at accelerating worm death.

Thus, dietary quebracho tannin may be an alternative to increasing dietary protein concentration, which increases the hosts' capacity to mount an immune response and expel the worm burden from the small intestine. These data suggest that feeding condensed tannins may be a suitable alternative to anthelmintics, especially in the areas of the tropics and subtropics. However, the potential anthelmintic properties of dietary condensed tannins may be limited to parasites that are in direct contact with digesta and/or feed on intestinal contents, mucus and mucosal cells. Similarly, the diverse nature of condensed tannins may result with anthelmintic properties being restricted to a specific condensed tannin structure.



## ABBREVIATIONS

|                |                                      |
|----------------|--------------------------------------|
| A              | Absorbance                           |
| A.B.V.         | Apparent biological value            |
| A.D.           | Apparent digestibility               |
| BSA            | Bovine serum albumin                 |
| CP             | Crude protein                        |
| CV             | Coefficient of variance              |
| d.f.           | Degrees of freedom                   |
| DM             | Dry matter                           |
| EDTA           | Ethylenediaminoethanetetracetic acid |
| ELISA          | Enzyme linked immunosorbant assay    |
| EPG            | Eggs per gram                        |
| GH             | Growth hormone                       |
| GI             | Gastrointestinal                     |
| GST            | Glutathione- <i>S</i> -transferase   |
| H              | High protein diet                    |
| HBSS           | Hanks' balanced salt solution        |
| Ig             | Immunoglobulin                       |
| L              | Low protein diet                     |
| L <sub>3</sub> | Third stage infective larvae         |
| N              | Nitrogen                             |
| OD             | Optical density                      |
| P.E.R.         | Protein efficiency ratio             |
| p.i.           | post infection                       |
| PBS            | Phosphate buffered saline            |
| PEG            | Polyethylene glycol                  |

|          |  |
|----------|--|
| PRP      | Proline rich salivary protein                    |
| PVP      | Polyvinylpyrrolidone                             |
| QT       | Quebracho tannin                                 |
| eQT      | Extracted quebracho tannin                       |
| uQT      | Untreated quebracho tannin                       |
| Rubisco  | Ribulose-1, 5-bisphosphate carboxylase/oxygenase |
| s.e.     | Standard error                                   |
| s.e.d.   | Standard error of difference                     |
| Tris     | Tris (hydroxy methyl) animo methane              |
| Tween-20 | Polyoxyethylene-sorbitan monolaurate             |

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## PUBLICATIONS

Some of the work presented in this thesis has been published, as indicated below.

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BUTTER, N.L., DAWSON, J.M., WAKELIN, D. AND BUTTERY, P.J. (1999) Dietary tannins acting as anthelmintic agents? *Proceedings of the IX International Symposium of Ruminant Physiology* (Abstract)

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# **CHAPTER 1**

## **INTRODUCTION**

### **1.1 GENERAL INTRODUCTION**

A major constraint on livestock production throughout the world is the infection of animals by helminth parasites (Gill & LeJambre, 1996). Chemicals, in the form of anthelmintic drenches, are extensively used to prevent animal mortality and maintain acceptable levels of production. The continued over-use and mis-use of these chemicals has enabled parasites to develop resistance as fast or faster than the pharmaceutical industry can develop new anti-parasitic drugs (Gill & LeJambre, 1996). For example in New Zealand in 1990, anthelmintic resistance was recorded to all three currently available drench families and was predicted to increase (Bailey, 1991). Increasing drug resistance, consumer concerns for chemical residues in meat and potential adverse environmental effects (Wall & Strong, 1987) have encouraged research to develop alternative strategies to control helminthiasis in livestock. Such alternative methods for parasite control include grazing strategies, selective breeding programs, worm vaccines, worm control using copper therapy, biological control, supplementary feeding and ethno-veterinary preparations and condensed tannins (Waller, 1999). These approaches, possibly with minimal, strategic anthelmintic use could lead toward economically and environmentally sustainable parasite-control systems.

### **1.2 PARASITISM AND THE HOST**

A parasite, as defined by the Concise Oxford English Dictionary (Allen, 1990), is an organism living in or on another and benefiting at the expense of the other, while Crofton (1971) proposed that parasites are those organisms which are potentially capable of killing their host. Parasites are metabolically dependent, directly or



indirectly, on the host to some degree. Dependence can include developmental stimuli, nutritional materials, digestive enzymes and control of maturation (Smyth, 1994).

Parasites can be broadly segregated as ectoparasites or endoparasites. Ectoparasites are organisms that live on the outside of their host attached to the skin, feathers, hair, follicles etc (Smyth, 1994). Ectoparasites from the phylum Arthropoda belong to the class Arachnida, mites and ticks, or the class Insecta including fleas, lice and flies. Endoparasites live within their hosts, inhabiting such areas as the gastrointestinal (GI) tract, body cavity, lungs or other tissues and are either protozoa or helminths (Smyth, 1994). The phylum Protozoa contains eukaryotic unicellular organisms being members of one of two groups, the Sarcomastigophora (amoebae and flagellates) and the Sporozoa (coccidia and malaria). Helminth parasites can be members of one of three phyla: Platyhelminthes including the trematodes (flukes) and cestodes (tapeworms); Nematoda, commonly called 'round worms' or Acanthocephala which shows similarities with both Platyhelminthes and Nematoda. Helminths are diverse in structure, physiology and behaviour. They use many different life cycle modes, varying from one-, two- or three-host life cycles. (Whitfield, 1993). A number of helminth parasites of sheep which have veterinary importance are shown in Table 1.1

GI parasites are probably the most numerous and widespread of all endoparasitic organisms. Both man and animal are susceptible to infection that may cause severe disease and ultimately death if not controlled (Wakelin, 1978). GI parasitic infection, clinical and subclinical, is characterised by impaired production, poor growth rates in young animals or bodyweight loss in older animals. Further changes include alteration of body composition, reduced milk and wool production including fleece quality, and poorer reproductive performance (Parkins & Holmes, 1989). Table 1.2 gives an indication of the loss of productivity in the ovine host when exposed to subclinical infections (Poppi *et al.*, 1990).



**Table 1.1** Some important helminth parasites of sheep

| Site            | Species                                       |
|-----------------|---|
| Abomasum        | <i>Haemonchus contortus</i>                   |
|                 | <i>Teladorsagia (Ostertagia) circumcincta</i> |
|                 | <i>Trichostrongylus axei</i>                  |
| Small intestine | <i>Nematodirus battus</i>                     |
|                 | <i>Trichostrongylus colubriformis</i>         |
|                 | <i>Trichostrongylus vitrinus</i>              |
|                 | <i>Cooperia curticei</i>                      |
|                 | <i>Bunostomum trigonocephalum</i>             |
| Large intestine | <i>Oesophagostomum venulosum</i>              |
|                 | <i>Oesophagostomum columbianum</i>            |
|                 | <i>Chabertia ovina</i>                        |
|                 | <i>Tirchuris ovis</i>                         |
| Liver           | <i>Fasciola hepatica</i>                      |

Taken from Anderson (1982)

**Table 1.2** Typical production losses due to endoparasitic infection of sheep

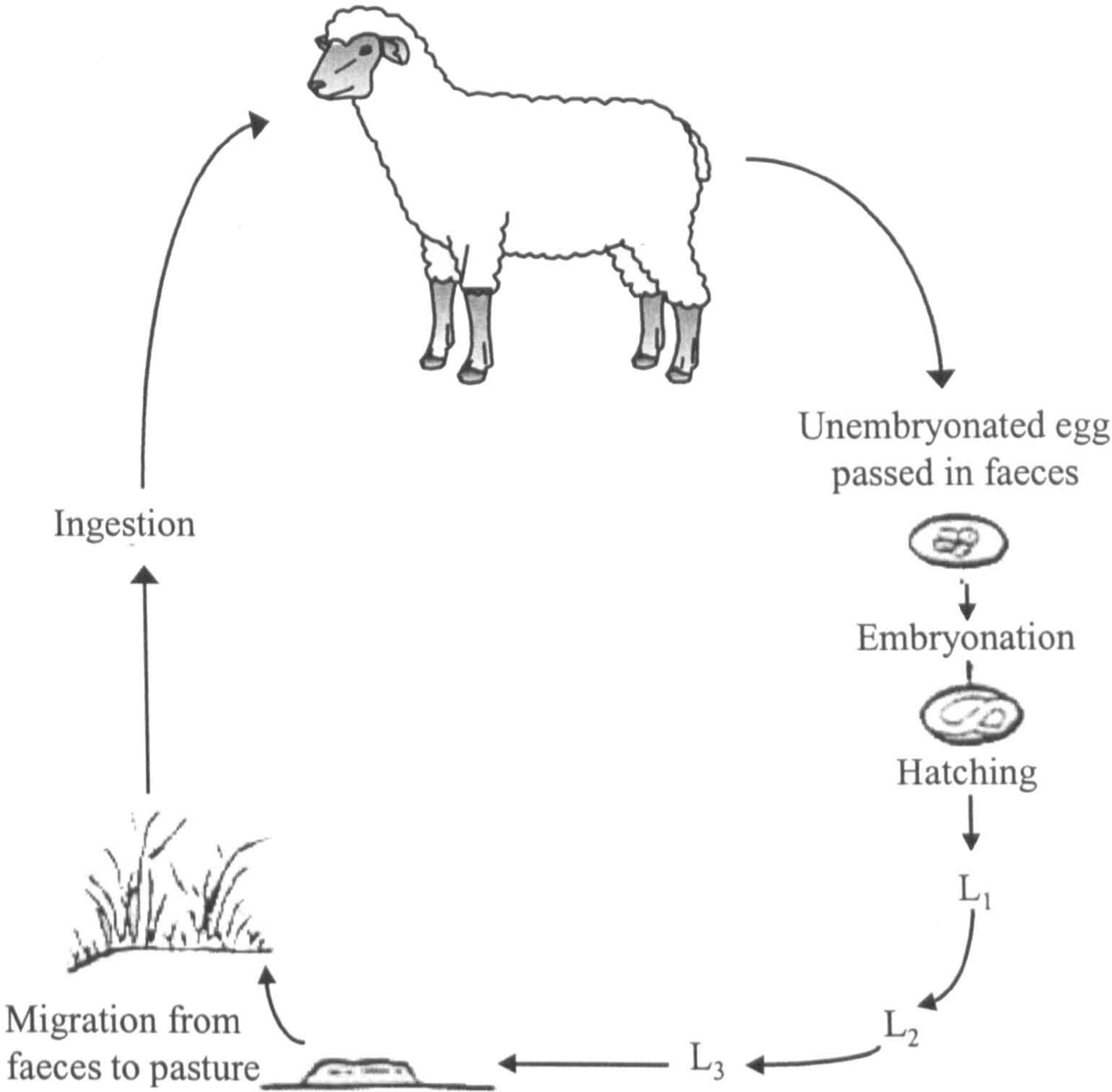
| Production parameter | % reduction |
|----------------------|-------------|
| Liveweight gain      | 40          |
| Feed intake          | 6-30        |
| Wool production      | 40          |
| Milk production      | 15          |

Figures taken from Poppi *et al.* (1990).

**1.2.1 Nematode life cycle and time course of infection**

Ruminant trichostrongyloid nematodes generally have similar life cycles (Figure 1.1). Eggs are passed in the faeces and develop into larvae that grow and moult twice to produce the infective third stage larvae (L<sub>3</sub>). L<sub>3</sub> show negative geotropic responses, causing them to migrate from the faecal pat onto the herbage, increasing their chance of being consumed by a passing host. Once ingested the larvae undergo two further moults to give the egg-laying adults (Parkins & Holmes, 1989; Smyth, 1994). The adult worms establish in the abomasum or intestine and either attach to the mucosa, leaving the body of the worm dwelling in the lumen or burrow into the mucosa. Experimental infection designed to investigate the aetiology of different infections can either be via the administration of a massive single dose of L<sub>3</sub> or trickle infections, where small doses of L<sub>3</sub> are administered daily or two to three times a week. The latter of these two approaches most closely simulates infection that animals are exposed to in the field. *Trichostrongylus colubriformis* infection of sheep follows the typical lifecycle outlined above (Figure 1.1). The host ingests infected pasture and the adult worm burden establishes in the small intestine. The presence of the parasite stimulates the immune system of the host. Host immune responses to nematodes are capable of reducing or terminating existing infections and also preventing re-infection (Cox, 1993). In a study by Kimambo *et al.* (1988) lambs were dosed daily with 2500 L<sub>3</sub> *T. colubriformis* for 34 weeks. Eggs were first

**Figure 1.1** Simplified lifecycle of intestinal nematodes of ruminants



L<sub>1</sub>, L<sub>2</sub> and L<sub>3</sub>, three larval stages  
adapted from Parkins & Holmes (1989)



seen in the faeces 3 weeks after initial infection and increased to a peak around week 5. Egg counts decreased to negligible levels by weeks 12-14 of dosing due to the development of host immunity against the parasitic infection. Laboratory animal model systems have been used extensively to determine the mechanism associated with immunity to GI nematodes (Lloyd & Soulsby, 1987). The life cycles of these parasites can vary from those seen in the ruminant. *Nippostrongylus brasiliensis* is a natural trichostrongyloid nematode of rats where infection is percutaneous. *N. brasiliensis* L<sub>3</sub> infect the host via skin penetration, enter the blood stream and pass through the heart to the lungs. The larvae moult and the fourth-stage larva are carried up the bronchi and trachea finally passing down the pharynx to the intestine where the final moult takes place to give the adult worms (Figure 1.2). After initial infection eggs are first seen in the faeces by day 5, reaching a peak on day 6-7 and decrease to negligible levels by day 11-12 post infection (Smyth, 1994).

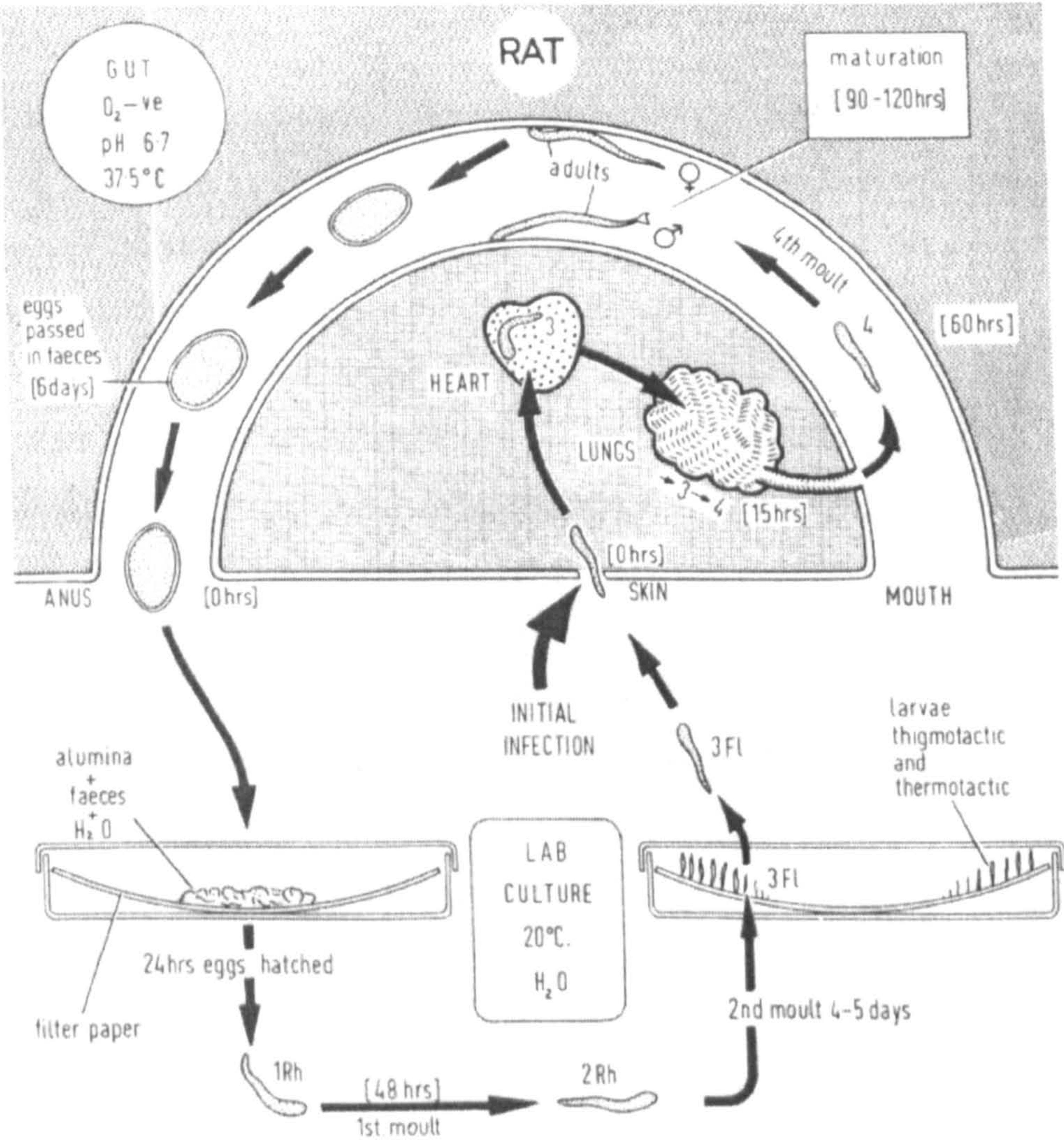
## 1.2.2 Host immunity

### 1.2.2.1 Primary infection

Trickle infections of the parasite-naïve host may initiate a ‘self cure’ response or spontaneous expulsion that expels the adult worm population from the intestine. L<sub>3</sub> form an established adult egg-producing population and then, quite suddenly, the host undergoes a series of intestinal changes resulting in worm expulsion. Resistance of the host to subsequent challenge infections is relatively strong, although can decrease over time (Wakelin, 1994). The mechanisms of expulsion are complex and not fully understood. Factors, which can affect the expulsion of a primary infection, include the level of infection, age, physiological status and genetic factors of the host (Lloyd & Soulsby, 1987). The protective responses of the host are associated with an inflammatory response of the GI mucosa leading to increased numbers of mucosal mast cells, globule leukocytes and eosinophils (Miller *et al.*, 1985; Douch *et al.*, 1986; Rothwell, 1989). Elevated concentrations of circulating antibodies against the parasite (Dawkins *et al.*, 1988; Adams *et al.*, 1989) and increased concentrations of antibodies in intestinal mucus also play a protective role (McClure *et al.*, 1992). The



**Figure 1.2** Life cycle of *Nippostrongylus brasiliensis* as carried out in the laboratory



Taken from Smyth (1994)



proliferation of goblet cells and increased mucus production have also been associated with worm expulsion (Miller & Nawa, 1979; Douch, 1990). These responses are T-cell dependent. The dependence of spontaneous expulsion on the presence of T-cells has been observed where athymic or T-depleted animals have been unable to develop immunity against parasitic infection (Lloyd & Soulsby, 1987).

#### *1.2.2.2 Challenge infection*

Re-infection of the primed host induces rapid expulsion of the parasite. The majority of incoming *T. colubriformis* L<sub>3</sub> are expelled from the immune host within 8h and *N. brasiliensis* within 4h of infection (Rothwell *et al.*, 1978; Miller *et al.*, 1981). Rapid expulsion of challenge infections is characteristic of immediate hypersensitivity reactions and the release of anaphylactic mediators of inflammation from local mucosal mast cells (Miller, 1984, Lloyd & Soulsby, 1987). Worm loss can be induced by administration of inflammatory mediators and prevented by antagonists. Nematode antigens bind to IgE-sensitised mast cells causing the release of chemical mediators that adversely affect nematode establishment (Rothwell, 1989). Immune sheep have increased concentrations of parasite-specific IgG<sub>1</sub> and IgA, histamine and anti-larval activity in mucus (Harrison *et al.*, 1999). Antibody in mucus and intestinal contents may be derived from local production or plasma leakage due to increased vascular permeability from mediators released by mast cells (Steel *et al.*, 1990). Harrison *et al.* (1999) hypothesised that immune mucus could prevent L<sub>3</sub> establishing in the intestine. L<sub>3</sub> may be physically prevented from penetrating the mucus layer due to increased viscosity, temporary paralysis by chemical mediators and/or entrapment of L<sub>3</sub> by antibody or glycoproteins in mucus. Products of mucosal mast cells, involved in the hypersensitivity responses, are known to influence goblet cells, the secretors of mucin (Specian & Oliver, 1991). Corticosteroid treatment prevents mucosal mast cell hyperplasia and the administration of mucolytic reagents inhibits worm expulsion (Miller & Nawa, 1979; Miller & Huntley, 1982) further supporting the importance of mucus in expulsion of nematodes. Globule leukocytes are closely related to, and may possibly be derived from, mucosal mast cells



(Huntley *et al.*, 1984). It is possible that mucus antiparasite substances are produced by these cells (Douch, 1990; Douch *et al.*, 1986).

Peripheral eosinophilia is one of the hallmarks of helminth infection, associated with the expulsion of the parasite from the gut (Dawkins *et al.*, 1989). Tissue eosinophilia, as blood eosinophilia, is associated with the responsiveness of sheep infected with *T. colubriformis* (Rothwell *et al.*, 1993), and has been implicated in the rejection of worms from the gut during challenge infections (Stevenson *et al.*, 1994). Datta *et al.* (1998) suggested that increased numbers of tissue and circulating eosinophils could provide an index of protective immune response of animals against helminth parasitism.

### **1.2.3 Gastrointestinal parasitic infection on host nutrition**

It has been established that the acquisition of immunity to parasitic infection can be altered by the nutritional status of the host (Wan *et al.*, 1989), where the nutritional status of the animal influences the pathogenesis of the infection (Gibson, 1963; Lunn *et al.*, 1988). Animals subjected to marginal levels of nutrition are more susceptible to infection leading to greater reductions in productivity (Niezen *et al.*, 1996). It has been well documented that animals receiving a high plane of nutrition, particularly the supplementation of dietary protein, are able to withstand some of the debilitating effects associated with parasitism (Poppi *et al.*, 1986; Abbott *et al.*, 1988; Bown *et al.*, 1991b; Kyriazakis *et al.*, 1994; van Houtert *et al.*, 1995 a, b, 1996; Donaldson *et al.*, 1997).

#### ***1.2.3.1 Effect of parasitic infection on feed intake***

Parasitic infection often results in reduced feed intake or inappetence of the host. The development of inappetence during parasitic infection is one of the major factors contributing to the reduced performance of the infected host (Coop & Holmes, 1996). The loss of appetite appears paradoxical as increased metabolic and

nutritional demands placed upon the host due to infection would be expected to be accompanied by compensatory feed intake (Kyriazakis *et al.*, 1998). The development of inappetence is often associated with the attainment of sexual maturity of the worms. Inappetence in sheep infected with *T. colubriformis* commences 3-4 weeks after initial infection, coinciding with the emergence of nematode eggs in the faeces indicating sexual maturity of the worm burden (Kyriazakis *et al.*, 1994; 1996b). Inappetence is also observed in the *N. brasiliensis*-rat model when larvae are migrating through the lungs (Crompton *et al.*, 1981) and when established in the small intestine (Horbury *et al.*, 1995). Normal feed consumption appears to return once the host has developed immunity to the infection (Kyriazakis *et al.*, 1996a) or almost immediately after the administration of anthelmintics removing the worm burden from the intestine (Kyriazakis *et al.*, 1998). Several concepts describing the mechanisms involved in inappetence have been postulated including abdominal pain (Miller, 1979), hormonal disruption (Symons, 1985; Dynes *et al.*, 1990, 1998) and feed aversion (Keymer *et al.*, 1983). In a recent review by Kyriazakis *et al.* (1998) two probable hypotheses explaining the occurrence of inappetence during parasitic infection were outlined. One hypothesis involved the promotion of the immune response by anorexia. Excesses of zinc, selenium and vitamins A and E can reduce the immune response of an animal (Chandra, 1993). Thus inappetence may have an 'immunopromotory' role. The second hypothesis links inappetence to increased diet selection. Reduced consumption of infective feed and the intake of alternative feedstuffs could reduce parasitic infection, particularly if the alternative feed might alter the GI tract making it unfavourable for parasite existence or contain antiparasitic components. Both of these hypotheses require further investigation but the development of inappetence may be an important strategy implemented by the host to reduce disease.

#### *1.2.3.2 Effect of parasitic infection on protein and energy utilisation and metabolism*

Parasitic infection causes GI tissue damage resulting in an increase of protein turnover and energy utilisation (Sykes & Coop, 1976; Poppi *et al.*, 1986; Bown *et al.*, 1991b). The development of immunity against the worm burden is also costly in terms of protein and energy (Kambara *et al.*, 1993). Increased demand for protein



and energy by the host to repair tissue damage and to mount an immune response is further exaggerated by the development of inappetence.

Digestion and absorption of protein and energy are generally not compromised by abomasal and anterior small intestinal parasitic infection (e.g. *T. circumcincta* and *T. colubriformis*) as the main site of protein digestion and absorption occurs in the distal small intestine beyond the site of infection (Bown *et al.*, 1991a). Balance studies have reported reduced nitrogen retention following GI parasitic infection. Increases in urinary nitrogen imply a reduction in utilisation efficiency of absorbed amino acids (Parkins & Holmes, 1989). Increased faecal nitrogen has been attributed to increased endogenous protein secretions, blood, plasma, mucin and sloughed cells into the gut lumen which are only partially reabsorbed distally along the tract (Kimambo *et al.*, 1988; Bown *et al.*, 1991a).

The development of resistance in sheep to GI parasites may be dependent on protein status (Bown *et al.*, 1991b; Kambara *et al.*, 1993). In contrast dietary protein supply appears to have no influence on the ability of the host to withstand initial infection (Coop, 1998). Bown *et al.* (1991b) infused casein, glucose or saline into the abomasum of sheep infected daily with 3000 *T. colubriformis* L<sub>3</sub> for 6 or 12 weeks. Worm burdens at 6 weeks were not affected by infusions of casein, glucose or saline. Data at 12 weeks showed that worm burdens recovered from casein-infused sheep were 50% lower compared to sheep infused with glucose or saline. This indicates that additional 'protected' protein supply, but not energy, can improve the hosts ability to expel the worm burden but has no bearing on the rate of establishment of infection in the parasite-naïve animal. An enhanced rate of worm expulsion was also observed in *T. colubriformis*-infected sheep fed hay supplemented with 0, 50 or 100g fish meal/day, while no effect was seen on the rate of worm establishment. Additional protein availability can elevate circulating concentrations of eosinophils and intestinal mast cell protease concentration, further supporting the enhancement of the immune system by increased dietary protein (van Houtert *et al.*, 1995b). Kambara *et al.* (1993) studied the influence of age and dietary protein on the resistance of lambs to *T. colubriformis*. Responsiveness of young lambs was



improved with dietary protein supplementation. T cell responses to mitogens and L<sub>3</sub> antigens, a measure of the immune status, were only well developed in older sheep fed supplementary protein. The negative correlation in high protein-fed young lambs indicates that these mechanisms are unlikely to play a major role in the development of resistance in parasite-naive young lambs. A recent study by Wallace *et al.* (1998) observed that the supplementation of urea (non-protein nitrogen degraded in the rumen) improved lamb's resilience to *H. contortus* infection, through increased liveweight gain. Resistance to infection was not affected by urea supplementation compared to control-fed lambs, faecal egg counts and worm burdens being similar between diets. This indicates that the supplementation of 'protected' protein sources or post-ruminal protein infusion promotes the acquisition of immunity against established worm burdens but increased rumen degradable protein sources such as urea provide no benefits with respect to improved immunity.

#### *1.2.3.3 Effect of parasitic infection on mineral metabolism*

Parasitic infection can lead to impairment of skeletal growth and mineralisation in sheep (Sykes & Coop, 1976). Phosphorus absorption and retention is reduced by small intestinal parasitism of sheep (Poppi *et al.*, 1985; Bown *et al.*, 1989) but not by abomasal infection (Wilson & Field, 1983). Coop & Field (1983) observed increased liveweight gain, reduced faecal egg counts and worm burdens at slaughter by increasing the phosphorus concentration of the diet of lambs infected daily with 2500 *Trichostrongylus vitrinus* L<sub>3</sub>, suggesting a role of phosphorus in the development of immunity to trickle infections.

The increased endogenous loss of calcium following infection, depressed plasma concentrations and evidence for compensatory absorption of calcium distal to the site of infection suggest that GI parasitic infection can affect the hosts ability to absorb calcium (Poppi *et al.*, 1985; Bown *et al.*, 1989). Reduced skeletal growth due to phosphorus deficiency would decrease the demand for calcium and may prevent calcium deficiency being of major importance. Abomasal infections can reduce copper absorption due to an elevation in abomasal pH affecting copper solubility

(Bang *et al.*, 1990). Reduced worm burdens and increased mucosal mast cells have been observed following the supplementation of dietary molybdenum in *H. contortus*-infected sheep, suggesting that molybdenum may enhance the immune response of the host (Suttle *et al.*, 1992). Cobalt deficiency has resulted in increased faecal egg counts and pepsinogen concentrations in *T. circumcincta* infected lambs (Ferguson *et al.*, 1989)

### 1.3 PLANT POLYPHENOLICS

#### 1.3.1 Occurrence

Tannins are phenolic compounds capable of forming stable complexes with protein and other macromolecules (Mangan, 1988). They are secondary metabolites that occur naturally in a variety of plants. The concentration of tannins present in plant tissue is often elevated in response to stress such as infection by bacteria and viruses, environmental stress including poor rainfall, light, temperature, soil fertility or death of plant tissue (Mueller-Harvey & McAllan, 1992; Van Soest, 1994). The nature, content and location of tannins vary considerably among plants. Tannins are regularly found in tree leaves, browse species and herbaceous legumes, which are important sources of feed for ruminants in arid and semi-arid regions (Kumar & Vaithiyanthan, 1990). These forages are often important sources of forage for ruminants during prolonged dry seasons, when pasture grasses and legumes are in short supply (D'Mello, 1992).

#### 1.3.2 Conventional classification

Tannins are commonly divided into two groups, hydrolysable tannins and condensed tannins. Hydrolysable tannins are water-soluble polyesters of phenolic acids and glucose or quinic acid and are susceptible to acid and enzymatic hydrolysis.



Condensed tannins are formed by the condensation of flavan-3-ols and produce coloured anthocyanidins on treatment with acidic alcohol and so are also named proanthocyanidins (reviewed by Mueller-Harvey & McAllen, 1992). Several groups of tannins have been identified which do not fit into either category. Table 1.3 identifies chemical preparations and some plants that contain predominantly hydrolysable or condensed tannins. Generally tree leaves and browse contain both types of tannins. It must be recognised that tannins are a diverse heterogeneous group of compounds and their ability to complex with other molecules is dependant on their structure (degree of polymerisation and molecular weight; Barahona *et al.*, 1997). Hagerman & Butler (1981) reported that the specificity of a tannin for a protein is also dependent on the conformational structure of that protein including size and charge on the protein molecule. Furthermore, Asquith & Butler (1986) reported that protein affinity was also a function of tannin chain length, so the formation of the tannin-protein complex is specific to both tannin and protein conformation. There are a wide variety of tannins and their structure is much more complex and diverse than the simplistic outline given above.

### **1.3.3 Dietary tannins and the monogastric animal**

Tannins in forage legumes can have a major impact on animal nutrition. The presence of large numbers of phenolic hydroxyl groups in condensed tannins enables tannins to form stable complexes with proteins and other macromolecules. In monogastric animals, feeding tannin-containing diets is generally associated with adverse effects on animal performance. This arises from reduced nutrient availability either directly, as a result of tannin binding, or through alterations to the physiology of the animal, causing inappetence, intestinal mucosal breakdown and at toxic concentrations, degeneration of different organs in the body (Makkar *et al.*, 1987).

**Table 1.3** Predominant tannin types of various plant tissue and chemical preparations

| Containing hydrolysable tannins | Containing condensed tannins |
|---------------------------------|------------------------------|
| <i>Plant tissue:</i>            | <i>Plant tissue:</i>         |
| Oak                             | Sainfoin                     |
| Salseed                         | <i>Lotus corniculatus</i>    |
| <i>Chemical preparations:</i>   | <i>Lotus pedunculatus</i>    |
| Tannic acid                     | <i>Rumex obtusifolius</i>    |
| Gallic acid                     | <i>Acacia aneura</i>         |
| Rutin                           | Sulla                        |
|                                 | Sorghum                      |
|                                 | Cottonseed                   |
|                                 | Quebracho                    |
|                                 | Grape seed                   |
|                                 | Faba-bean                    |



#### 1.3.3.1 Effect on feed intake

Feeds containing tannins are described as being astringent (the sensation caused by the formation of a complex between tannins and salivary glycoproteins). At low concentrations tannins give food 'bite' and 'character' and are responsible for the liking of tea and red wine. The astringent nature of foods containing high concentrations of tannins can reduce palatability, feed intake and thus animal productivity. In natural ecosystems different herbivores select plant material differing in tannin concentration, the accepted dietary tannin concentration of animals in their natural environment varies between species (Mole, 1989). Some animals can adapt to the presence of dietary tannins by producing salivary proline-rich proteins (PRPs) which bind to tannins in the mouth and thereby prevent tannin binding to dietary proteins. Mehansho *et al.* (1987) showed that PRPs have an affinity for tannin which is about 10-fold greater than that for the complex formed between tannin and BSA, further indicating specificity in the tannin-protein interaction. Long-term ingestion of dietary tannins can induce enlargement of the salivary glands (Van Soest, 1994). However, not all animals can secrete salivary PRPs. Rats, man and browsing species of animal such as deer or antelope secrete PRPs but in other species including sheep, cattle and hamsters, PRPs seem to either be lacking or salivary production is low (Austin *et al.*, 1989).

#### 1.3.3.2 Effect on animal performance

The presence of tannins in the diets of monogastric animals reduces performance mainly by depressing nutrient availability, and at high concentrations, through reduced feed intake. The capacity of tannins to form stable complexes with proteins accounts for most of their adverse effects. A negative correlation exists between tannin concentration and protein digestibility in monogastric diets. Many experiments have shown reduced liveweight gain and poor feed efficiencies when tannins have been included into the diet of chicks (Ahmed *et al.*, 1991; Mahmood & Smithard, 1993; Ortiz *et al.*, 1994), rats (Featherston & Rogler, 1975; Elkin *et al.*, 1990; Ortiz *et al.*, 1994) and pigs (Cousins *et al.*, 1981; Mitaru *et al.*, 1984;). In addition to reducing protein digestibility tannins are also capable of forming a complex with carbohydrates, minerals, vitamins, bacterial cell membranes and

enzymes involved in protein and carbohydrate digestion (Makkar *et al.*, 1987). Data demonstrating the negative effects of condensed tannins in monogastric diets includes a study by Jambunathan & Mertz (1973) where rats fed 6 varieties of sorghum with tannin concentrations ranging between 4.7 and 68.8 g/kg dry matter (DM). The protein efficiency ratio (average protein consumed/average gain) of the diet was reduced as tannin concentration increased. Mitaru *et al.* (1984) recorded a reduction in the digestibility of protein and individual amino acids at the terminal ileum of pigs fed sorghum grain (850g/kg diet) containing 4.7% condensed tannin. Similarly in growing rats and pigs, cottonseed tannins were shown to depress ileal protein digestibility (Yu *et al.*, 1996b). Yu *et al.* (1996a; b) postulated that bound cottonseed tannin in the diet (as opposed to free tannin) is solubilised by the acidic conditions of the stomach and is then available to react with proteins in the small intestine.

#### *1.3.3.3 Effect on the gastrointestinal tract*

Some tannins have been shown affect the functional state of the GI epithelium of monogastric animals which will impair nutrient absorption and utilisation. In some cases, tannins (or their metabolites) have been shown to be absorbed from the GI tract, with degradation products appearing in blood, urine and faeces (Butler *et al.*, 1986). Hydrolysable tannins (e.g. tannic acid) orally administered to monogastric animals have been reported to cause oesophageal and gastric oedema, haemorrhagic ulceration, erosion and mucus hypersecretion from the mucosa of the stomach and duodenum (Dollahite *et al.*, 1962; Mitjavila *et al.*, 1977). Intestinal mucus hypersecretion has also been reported from feeding condensed tannins to rats (Sell *et al.*, 1985) which will contribute to increased endogenous protein losses. Increased enterocyte proliferation might also be expected in tannin-fed animals. However, no increase in the fractional rate of protein synthesis in the duodenal mucosa was seen in tannin-fed rats (Dawson *et al.*, 1999). Intestinal brush border hydrolase activities (e.g. alkaline phosphatase and sucrase), which are believed to reflect the functional activity of the intestinal epithelium, have been shown to be reduced at the villus tip and along the length of the villus-crypt axis of the jejunum in rats fed grape seed tannins at 20 g/kg (Vallet *et al.*, 1994; Tebib *et al.*, 1994). This effect was



accompanied by an increase in  $^3\text{H}$ -thymidine incorporation in the middle of the crypt zone of the jejunum which suggests the rate of enterocyte proliferation was stimulated in the tannin-fed animals (Vallet *et al.*, 1994). Interestingly, these authors observed no effect of the tannin on alkaline phosphatase or sucrase activity in the duodenum. This was suggested to be due to the alkaline pH and/or the higher concentration of bile salts in the duodenal lumen compared with lower down the GI tract which counteracted the ability of tannins to reduce the activity of brush border hydrolases (Vallet *et al.*, 1994; Tebib *et al.*, 1994). Addition of pancreatic-biliary juice to isolated brush border membrane preparations incubated with tannins *in vitro* reversed the inhibitory effect of the tannins (Tebib *et al.*, 1994).

In monogastric animals, dietary condensed tannins from leaves of different fodder plants were shown to reduce the activity of trypsin and  $\alpha$ -amylase in the rat but in the *in vitro* situation, lipase activity was also decreased (Horigome *et al.*, 1988). Comparable results were also reported by Griffiths & Moseley (1980) and in cockerels by Mahmood & Smithard (1993). Fahey & Jung (1989) postulated that the capacity of tannins to inhibit digestive enzymes may be a function of:

- availability of dietary protein
- extent of the formation of a protein and tannin complex prior to ingestion
- relative amounts of different enzymes present
- order in which the tannin encounters the enzymes
- affinity of the enzymes to complex with the tannin.

van Leeuwen *et al.* (1996) postulated that protein digestion of pigs fed faba-bean tannins was limited by the decrease in aminopeptidase activity. In contrast, papers reviewed by Mueller-Harvey & McAllan (1992) suggest that the anti-nutritional effects of tannins are due to formation of a tannin-substrate complex and not enzymatic inhibition (Mole & Waterman, 1987; Blytt *et al.*, 1988). Faecal nitrogen is generally increased in both monogastric and ruminant animals consuming tannins while urinary nitrogen excretion is reduced. The increase in faecal nitrogen may arise from (i) binding of tannins to digesta protein thus reducing availability, (ii) reduced activity of digestive enzymes or (iii) by impairment of the functional activity of the

intestine. Increased secretion of endogenous proteins (salivary proteins, digestive enzymes, mucus or mucosal cells) may also contribute to increased faecal N excretion.

#### **1.3.4 Dietary tannin and the ruminant**

High concentrations of dietary tannin, generally considered to be above 60g/kg DM (Waghorn, 1990), have been reported to reduce feed intake in sheep by several workers. Pritchard *et al.* (1988) recorded a 40-50% reduction in feed intake in sheep fed between 2-11% *Acacia aneura* (mulga, containing condensed tannins) when compared to those animals supplemented with polyethylene glycol (PEG), which forms complexes with tannins in preference to other molecules such as dietary protein. Waghorn & Shelton (1995) estimated that as little as 55g condensed tannin/kg DM was sufficient to depress feed intake in sheep grazing *Lotus pedunculatus*, while concentrations below this level appear to have little or no effect on feed intake.

In ruminants the negative correlation of dietary tannins against protein digestibility is only seen at higher dietary inclusions. As will be discussed below, low inclusion rates can be beneficial. Feeding low concentrations (~10- 40g condensed tannin/kg DM, (Waghorn, 1990)) of some tannins to ruminants has been found to be beneficial. PEG forms complexes with tannins in preference to protein and its addition to tannin-containing feed therefore prevents the tannin from binding with dietary protein. Inclusion of PEG in tannin-containing forages has lead many researchers to conclude that productivity of ruminants can be increased by feeding low concentrations of condensed tannins. This is due to the capacity of condensed tannins to bind to dietary protein and protect the protein from rumen degradation resulting in increased post-ruminal protein supply (Waghorn *et al.*, 1987b). The tannin-protein complex is believed to dissociate in the abomasum leaving the protein available for post-ruminal absorption and hence releasing free condensed tannins that may be able to react with free protein further along the GI tract, as discussed later. The effect of



dietary tannins in ruminants can be divided into two sections, the first being the consequence of dietary tannins in the rumen and secondly post-rumen gut effects.

#### *1.3.4.1 Dietary tannin in the rumen*

In contrast to monogastric animals, feeding low concentrations of condensed tannins in the diet of ruminants (~10-40g/kg DM) has been reported to improve animal productivity. One popular mechanism which has been postulated for this response is the complex formed between soluble protein and tannin in the near neutral pH of the rumen preventing microbial degradation (Jones & Mangan, 1977; Barry & Manley, 1986). The formation of tannin-protein complexes in the rumen also results in an elevation of the rumen non-ammonia nitrogen pool and a decrease in rumen ammonia concentration (Table 1.4). This generally reduces ammonia losses from the rumen and urea losses in urine. Martin *et al.* (1985) demonstrated that a protein-tannin complex is stable *in vitro* in the pH range 5.6-7.0 but above or below this range, the complex dissociates. Once the complex passes through the rumen it becomes subjected to gastric (pH 2.5-3.5) and pancreatic (pH 8.0) secretions and dissociates making the protein available for absorption from the small intestine, increasing bypass protein flow (Figure 1.3).

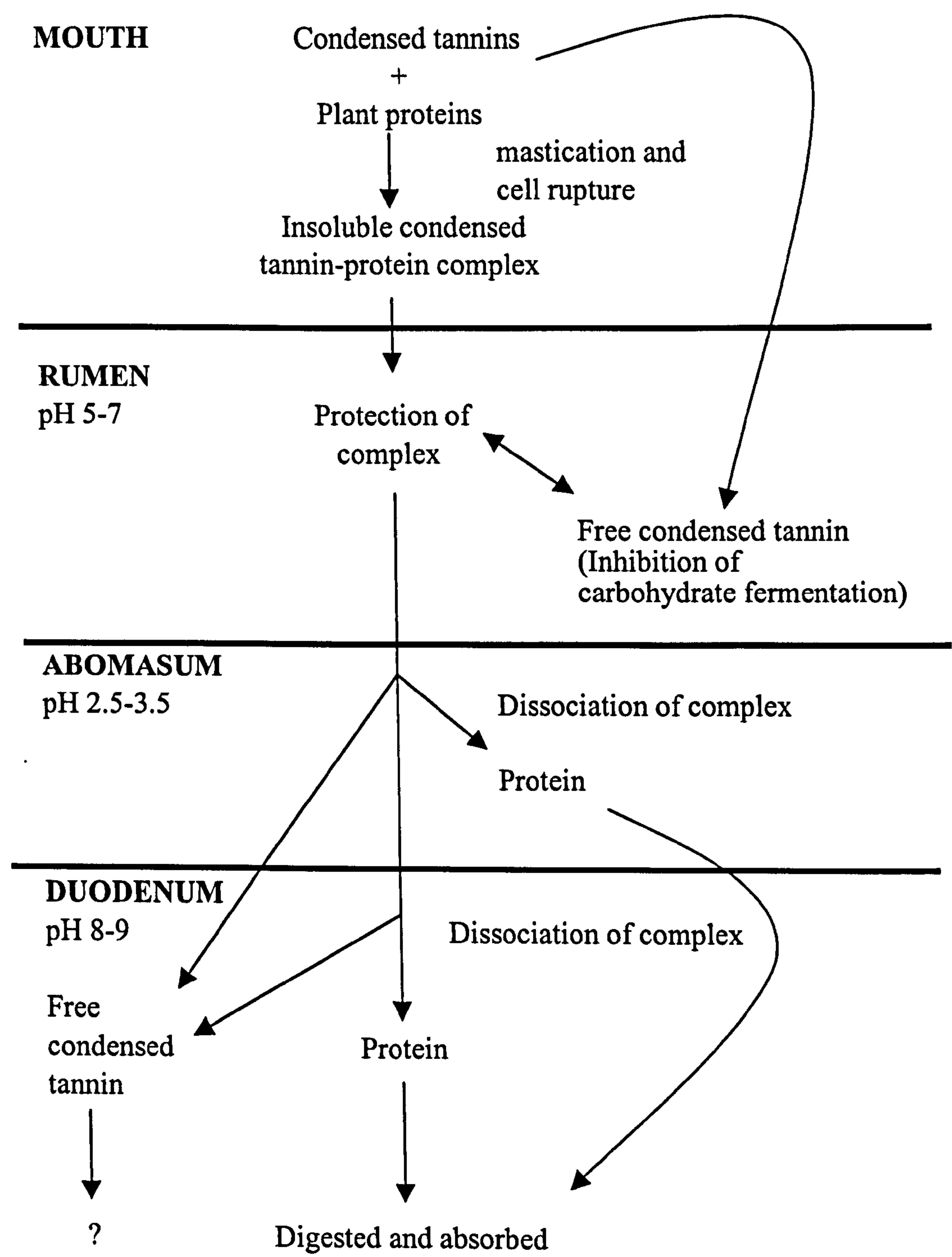
In some cases, the presence of dietary tannins has been suggested to increase saliva production (Van Soest, 1994). Increasing salivary flow to the rumen may elevate the levels of salivary urea present which rumen microbes can utilise for growth and multiplication. Thus there is some suggestion that tannins may improve animal productivity through improved urea recycling and utilisation resulting in increased microbial protein synthesis in addition to protection of dietary protein in the rumen. It should be noted that an increase in salivary production is not always apparent in tannin-fed animals. Waghorn *et al.* (1994a) found no evidence of increased salivary flow to the rumen in sheep fed *Lotus pedunculatus* (55g condensed tannin/kg DM) in comparison to PEG-fed animals. Studies reported by Beever & Siddons (1986)

**Table 1.4 Nitrogenous aspects of sheep fed condensed tannin forages and those complexed with PEG or containing negligible levels of condensed tannin**

|  | Reference  | Diet<br>(condensed<br>tannin/kg DM)    | Condensed<br>tannin-forage           | Condensed<br>tannin-forage<br>+PEG | Pasture<br>(neg. tannin) |
|--|--|--|--------------------------------------|------------------------------------|--------------------------|
| Rumen non-ammonia                        | Waghorn <i>et al.</i><br>(1994b)   | 55g                                    | 12.5                                 | 8.6                                |                          |
| Nitrogen pool<br>(g)                     | Waghorn <i>et al.</i><br>(1987b)   | ~35g                                   | 10.5                                 |                                    | 8.6                      |
| Rumen ammonia<br>Concentration<br>(mg/l) | Waghorn <i>et al.</i><br>(1994b)<br><br>Terrill <i>et al.</i><br>(1992a)<br><br>Waghorn <i>et al.</i><br>(1987b)<br><br>Barry <i>et al.</i><br>(1986b) | 55g<br><br>~45g<br><br>~35g<br><br>95g | 283<br><br>126<br><br>225<br><br>275 | 507<br><br>150<br><br><br>389      | <br><br><br><br>334      |



**Figure 1.3** Condensed tannins and protein protection in the rumen



Taken from D'Mello (1992)

indicated an increase in microbial protein flow to the duodenum after feeding moderate concentrations of condensed tannins in the diet of sheep fed sainfoin. Other studies however have reported reduced microbial protein flux to the duodenum of condensed tannin-fed animals (Waghorn *et al.*, 1987a; Waghorn *et al.*, 1994b). Often the effect of condensed tannin-containing forages are compared to pasture (~zero condensed tannin). These two forages may vary widely in nutritive content, and hence could alter microbial protein synthesis. Hume *et al.* (1970) demonstrated that microbial growth in the rumen linearly increases to a maximum of 50 g protein leaving the rumen each day when nitrogen intake was 9 g/day. Other nutrients, which can reduce bacterial growth and efficiency, include inadequate concentration of branched-chain fatty acids, ammonia and amino acids (Owens & Goetsch, 1986).

Reducing rumen degradability of nitrogen confers a further advantage to ruminants of feeding very low concentrations of tannins (<5g/kg DM) by preventing bloat. Bloat is a widespread condition affecting ruminants, particularly those grazing highly productive forages such as lucerne and clover (Mangan, 1988). It occurs when digestive gases become trapped in the rumen due to the formation of a stable and persistent foam that can lead to compression of the cardiovascular system and ultimately death. The foam formation is primarily the result of soluble protein, namely Fraction 1 plant leaf protein, being present in rumen liquor (Jones *et al.*, 1978), with lucerne, clovers and other productive forages releasing large quantities of soluble protein into the rumen environment. Kendall (1966) showed that condensed tannins from crude plant extracts were capable of inhibiting foam production *in vitro*. Forages which contain condensed tannins such as sainfoin, *Lotus corniculatus* and *Lotus pedunculatus* are generally viewed as non-bloating species (Jones *et al.*, 1970; Jones *et al.*, 1973; Clarke & Reid 1972). The supplementation of small quantities of condensed tannin herbage to the main forage has been also shown to be beneficial against bloat. Cattle fed a predominantly lucerne forage supplemented with approximately 10% dock (*Rumex obtusifolius*, ~20g condensed tannin/kg DM) did not bloat compared to those fed the dock-free herbage (Waghorn & Jones, 1989).



Not all of the effects of tannins are beneficial to ruminants although the magnitude of the deleterious effects may also vary with different tannins and concentration. Whilst the affinity of tannins for binding to dietary carbohydrates is considerably less than that observed for proteins, the presence of tannic acid or catechin *in vitro* nevertheless decreased the digestibility of starches by 9-17% (Deshpande & Salunkhe, 1982) and the amylolysis of several legume starches (Makkar *et al.*, 1987). Waldo (1973) demonstrated that the digestibility of starch in the rumen decreased with increased levels of tannins. Fibre digestion has also been shown to be reduced by dietary condensed tannins (Barry & Duncan, 1984, Yu *et al.*, 1995) while other workers have seen no reduction, possibly due to an extended residence time of feed in the rumen (Waghorn *et al.*, 1994a). Sheep fed a pelleted diet of dried grass containing quebracho tannin at 50 g/kg showed a reduction in apparent digestibility of neutral detergent fibre from 63.3% to 58.8 % after 2 weeks of feeding the diet (Dawson *et al.*, 1999). This reduction in fibre digestibility is believed to be largely due to inhibition of rumen microbial activity by tannins. Condensed tannins from *L. corniculatus* reduced the digestion of filter paper by *Fibrobacter succinogenes* (the most active rumen bacteria for cellulose digestion) to 45% when incubated with 300µg condensed tannin/ml and virtually no activity was observed at concentrations greater than 400µg condensed tannin/ml (Bae *et al.*, 1993). In a review Makkar *et al.* (1987) stated that tannins were reported to inhibit various bacterial enzymes involved in the metabolism of carbohydrates including cellulase, amylase and galactosidase. *In sacco* studies by Makkar *et al.* (1988) on the effects of predominantly hydrolysable tannins extracted from oak leaves on some rumen microbial enzymes in rumen fluid obtained from animals with no previous adaptation to tannin diets showed significant reductions in the activities of urease, carboxymethyl-cellulase and various ammonia-assimilating enzymes. The presence of tannins tended to affect the microorganisms that were tightly bound to the solid matrix more markedly than those in the more complex microbial compartment. Growth and proteolytic activity in 3 strains of rumen bacteria was reduced by the presence of condensed tannins from sainfoin *in vitro* (Jones *et al.*, 1994). A fourth bacteria *Prevotella ruminicola* was unaffected by the presence of the tannin and appeared to produce an extracellular material which protected the microorganism from the morphological changes observed in the other 3 strains. Chiquette *et al.*, (1988) showed that the



colonisation of leaf material by rumen bacteria was reduced if the tissues contained high levels of condensed tannins.

Total rumen protozoal counts have been observed to decline in ruminants fed tannin diets. Makkar *et al.* (1995a) reported a decrease in protozoal numbers when rumen fluid was incubated *in vitro* with concentrations ranging from 0.2 to 0.4 mg quebracho tannin/ml. Wang *et al.* (1994, 1996a) found that the condensed tannin in lotus had an adverse effect on protozoal growth, with PEG-supplemented sheep having higher numbers of protozoa in the rumen fluid. Some benefits have been reported where protozoal numbers are decreased. Bird & Leng (1985) postulated that a reduction in protozoal numbers is likely to be beneficial by increasing dietary protein available for intestinal digestion especially when the animal is maintained on low quality pasture. Protozoa are predatory on rumen bacteria, a reduction in protozoa is accompanied by an increase in the bacterial population. Results with tannin-containing diets are however not always consistent with the above; for example in a study carried out by Terrill *et al.* (1992a) an increase in protozoal numbers was recorded in sheep grazing sulla in comparison to control animals grazing pasture.

A reduction in fibre digestion in the rumen will reduce energy supply to rumen microbes, slowing their growth and hence microbial protein synthesis and microbial protein supply to the host animal. Barry & Manley (1984) observed depressed ruminal digestion of readily fermentable carbohydrate and hemicellulose in sheep fed *L. pedunculatus* (both at 46 and 106 g condensed tannin/kg DM). It was postulated that free condensed tannins were precipitating the extra-cellular microbial enzymes involved in the ruminal degradations of these dietary components. However, the presence of 60 g condensed tannin/kg DM in sainfoin did not appear to impair rumen digestion of carbohydrates (Ulyatt & Egan, 1979). The difference in the action of these two forages may be a consequence of the different molecular weights of the condensed tannin, sainfoin contains condensed tannins of a higher molecular weight (MW; 22 000) than those present in *L. pedunculatus* (MW; 7700). Low molecular weight condensed tannins tend to be more efficient at precipitating



protein and possibly inactivating enzymes involved in rumen carbohydrate digestion (Barry & Manley, 1984).

The production of volatile fatty acids *in vitro*, produced by fermentation of organic matter, have been shown to be reduced by tannins (Makkar *et al.*, 1995a) which would reduce energy substrates available to the animal. Changes in the molar proportions of the volatile fatty acids have also been reported with reduced acetate:propionate ratios. In those papers reporting improved productivity of animals fed tannin-containing diets, the capability of tannins to protect protein from rumen degradation must be greater than the deleterious effects of reducing carbohydrate digestion. At high condensed tannin concentrations the benefit of increased protein flow at the duodenum may be negated by reduced fibre digestion in the rumen and reduced voluntary intakes (Barry & Duncan, 1984; Barry *et al.*, 1986b).

Although some tannins may inhibit rumen microbial activity, especially at moderate to high concentrations, rumen micro-organisms are capable of rapidly metabolising many low molecular weight phenolic compounds (Parrinder *et al.*, 1993a, b) which may provide another adaptation mechanism for ruminants consuming tannin-rich diets. Makkar *et al.* (1995a) suggested that monomeric and dimeric flavanols are degraded by rumen microbes but larger oligomeric condensed tannins remain undegraded. Robbins *et al.* (1991) reported that ingested quebracho tannin was completely recovered from the faeces of mule and black bears, but in sheep only 75% was recovered in the faeces. It was suggested that the remaining 25% may have been metabolised in the digestive tract and absorbed. Mule and black bears secrete PRPs, while they are absent in the sheep, possibly reflecting the difference in recovery of tannins in faecal material. Scalbert (1991) has postulated that some micro-organisms, including some that exist in the rumen, can protect themselves from the negative effects of tannins. Rumen bacteria grown on *Lotus corniculatus* (containing condensed tannins) have been observed secreting glycoproteins which are not produced when grown on low-tannin varieties (Chiquette *et al.*, 1988). Makkar *et al.* (1995a) suggested that rumen micro-organisms may adapt to the presence of dietary tannins with prolonged feeding by inducing enzymes capable of

degrading tannins. However, no evidence to support this hypothesis was obtained in a study in which sheep were fed 50 g quebracho tannin/kg diet for 2 or 6 weeks (Dawson *et al.*, 1999).

#### *1.3.4.2 Dietary tannin and post-ruminal effects*

As discussed above, the tannin-protein complex which survives passage through the rumen is believed to dissociate in the acidic conditions of the abomasum releasing the protein which is then available for absorption in the lower GI tract. However free tannin is also released which presumably can react with other proteins including digestive enzymes and the gut mucosa (Jones & Mangan, 1977) in the less acidic conditions of the small intestine (Figure 1.3). Thus tannins surviving passage through the rumen may induce negative effects in the lower GI tract of ruminants similar to those reported in monogastric animals fed tannin-rich diets.

In ruminant studies, several researchers have recorded an increase in the net absorption of amino acids from the small intestine in tannin-fed animals but evidence suggests that the site of amino acid absorption is altered. Wang *et al.* (1996b) reported a reduction in the proportion of methionine and cysteine digested in the proximal part of the small intestine of sheep but measured a proportional increase in that digested in the last third of the small intestine. It is possible that the tannin-protein complex which dissociated in the acidic conditions of the abomasum reformed as the digesta passed into the small intestine as the pH increased, thus impairing amino acid absorption in the proximal intestine. As the complex passes further along the gut the pH continues to rise and reaches pH 8.0 and above as it nears the last third of the small intestine which may allow the complex to dissociate yet again. Evidence presented by Wang *et al.* (1996b) suggested that some tannin-protein complexes were still present at the terminal ileum, with further dissociation in the large intestine probably due to microbial fermentation. Values for amino acid absorption from the small intestine reported by Wang *et al.* (1996b) and McNabb *et al.* (1993) are shown in Table 1.5. The variation is likely to be dependant on the



**Table 1.5** Apparent absorption (g/g eaten) of sulphur amino acids in sheep fed condensed tannin forage and those complexed with PEG

| Reference                   | Diet<br><br>(condensed tannin/kg DM) | Condensed tannin-<br><br>forage | Condensed tannin-forage<br><br>+ PEG |
|-----------------------------|--------------------------------------|---------------------------------|--------------------------------------|
| Wang et al.<br><br>(1996b)  | 20g extractable                      | Methionine<br><br>0.49          | 0.72<br><br>0.48                     |
| McNabb et al.<br><br>(1993) | 55g extractable                      | Methionine<br><br>Cysteine      | 0.75<br><br>0.42                     |
|                             |                                      |                                 | 0.56<br><br>0.38                     |

concentration of tannin in the diet and the molecular weight and structure of the tannins. Several workers have reported an increase in the apparent absorption of amino acids from the small intestine (Waghorn *et al.*, 1987b; Lee *et al.*, 1995). Caution should be taken when evaluating these results; the presence of tannins at least in monogastric diets is associated with increased endogenous secretions, as covered later in this review, and this elevation is likely to influence apparent absorption values for individual amino acids. In other studies, Waghorn *et al.* (1994a) reported a 15-20% reduction in the proportion of amino acids absorbed from the small intestine of sheep fed *L. corniculatus* but this was compensated for by an increased nitrogen flow to the intestine. It was postulated that the mechanism through which condensed tannins may reduce amino acid absorption could be due to condensed tannins inhibiting endogenous enzyme activity or by associating with the intestinal mucosa. In a review by Makkar *et al.* (1987) dietary tannins were reported to inhibit various enzymes in the intestinal mucosa. Alkaline phosphatase and 5'-nucleotide phosphodiesterase activities were reduced in cattle fed sorghum tannins. Egan & Ulyatt (1980) observed an increased N retention in sheep fed sainfoin (condensed tannin ~10-11%) when compared with those fed white clover and ryegrass (condensed tannin ~zero). While apparent and true digestibility of protein was lower on the sainfoin diet the reduction in the loss of urinary N was sufficient to compensate for the increased faecal N output. Total nitrogen digestibility values of sheep grazing a condensed tannin forage or those fed the forage where the condensed tannins have been complexed by PEG are shown in Table 1.6. The addition of PEG to forages containing condensed tannins increases the apparent nitrogen digestibility of the feed.

Histological evidence from samples obtained from the GI tract of sheep fed quebracho tannin suggest that tannins surviving passage through the rumen can cause morphological changes and impair the functional integrity of the intestine (Dawson *et al.*, 1999). While there was little evidence of morphological changes in the duodenum of tannin-fed sheep, epithelial degeneration and ulceration with increased mucosal histiocytes was observed in the jejunum and ileum suggesting that the tannin may be locally toxic to surface epithelium in this region of the GI tract. In



Table 1.6 Apparent digestibility of nitrogen of lambs grazing condensed tannin forages with or without the supplementation of PEG

| Reference              | Diet<br><br>(condensed tannin/kg DM) | Control | PEG  |
|------------------------|--------------------------------------|---------|------|
| Waghorn et al. (1997)  | ~10g                                 | 0.76    | 0.80 |
| Wang et al. (1994)     | 23g                                  | 0.72    | 0.80 |
| Waghorn et al. (1987b) | 22g                                  | 0.70    | 0.78 |

addition, there was some evidence that quebracho tannin was taken up by Peyer's patches, which could lead to an immune response. It appeared that the tannin was probably phagocytosed by lamina propria macrophages and persisted there for some time. Uptake of tannins or their metabolites has been demonstrated previously (Butler *et al.*, 1986; Bravo *et al.*, 1994) and could explain how tannin ingestion can alter the activity of some tissue enzymes (e.g. hepatic uridine diphosphate glucuronyltransferase, Sell & Rogler, 1983) and thus influence post-absorptive metabolism.

Some tannins have been reported to alter body composition of ruminant animals. Purchas & Keogh (1984) reported that lambs fed fresh forages containing high levels of condensed tannins have a lower carcass fat content. This may be due to poor utilisation of ingested nutrients that would result in less energy available for fat deposition. There may also be other explanations. Barry *et al.*, (1986a) reported that adipose tissue from sheep fed condensed tannins from *L. pedunculatus* had consistently higher rates of lipolysis with a consequent reduction in the lipogenesis:lipolysis ratio. It was postulated that the change in adipose tissue metabolism was mediated by growth hormone (GH) as circulating levels were higher in tannin-fed sheep (Barry *et al.*, 1986a). Increased plasma GH concentrations could be due to tannins inactivating gut-wall proteins thus stimulating secretion (Barry *et al.*, 1986a) or could be due to reduced nutrient availability which is believed to reduce metabolic clearance of GH, possibly through increased protein binding which increases GH half-life (see Bass *et al.*, 1992). Plasma GH concentrations are often elevated in nutritionally-deprived animals (Dawson *et al.*, 1998). Changes in rumen fermentation by tannins could also contribute to reduced fat deposition in ruminants. As mentioned above some reports have indicated that tannins alter the molar proportions of volatile fatty acids produced, increasing propionate proportion at the expense of acetate (Makkar *et al.*, 1995b) which is presumably related to the tannin-induced reduction in fibre digestion.



## 1.4 TOXICITY

The benefits of feeding dietary tannins are generally restricted to condensed tannins and whilst at high concentrations these may be detrimental, they are rarely toxic (Singleton & Kratzer, 1969). However, the converse is often true of hydrolysable tannins. In a review by Jansman (1993) it was stated that chicks fed hydrolysable tannins may suffer from fatty liver, necrosis of the liver and kidneys, varying degrees of desquamation in the surface epithelium and necrosis of the epithelial layer of the small intestine. Liver function has been shown to be altered in rats injected with tannic acid and high mortality and morbidity were observed in cattle and sheep fed dietary concentration of above 20% hydrolysable tannins. Microbial tannases present in the rumen hydrolyse these compounds to galloyl esters which are metabolised and absorbed across the rumen wall and subjected to further metabolism (Murdiati *et al.*, 1992). Condensed tannins are larger compounds and tend not to be metabolised and absorbed from the digestive tract, thus their detrimental effect to the animal is believed to be through reducing nutrient availability and some mucosal damage. However, Rittner & Reed (1992) reported an increased mortality of ruminants feeding on tropical tree legumes containing high concentrations of condensed tannins with a low dietary protein content.

## 1.5 DIETARY TANNIN AND PARASITIC INFECTION

### 1.5.1 Potential of dietary tannins to reduce parasitic infection

The control of GI parasites is still heavily reliant on the use of anthelmintic drugs. Not only is there increasing consumer concern over the presence of chemical residues in the food chain and general environment but their continued use and misuse is developing an ever accelerating state of anthelmintic resistance (Gill & LeJambre, 1996). One possible alternative to the heavy reliance that is placed on anthelmintics is the introduction of forage species capable of reducing parasitism,

solely or in conjunction with limited drug use. Chandel & Metha (1990) demonstrated that wild sage (*Lantana camara*) possesses nematocidal properties capable of acting as an effective control of a number of plant parasitic nematodes. Eucalyptus species containing Mannich bases (containing an aromatic ring side chain) fed to goats have proved to be effective anthelmintics against *H. contortus* but not *Teladorsagia* spp. (Bennet-Jenkins & Bryant 1996). The use of a nematode larval motility inhibition assay has shown certain plants to have anthelmintic properties against *T. colubriformis* (Lorimer *et al.*, 1996).

Parasite-infected lambs and sheep grazing condensed tannin-containing forages have been reported to have improved liveweight gain and reduced faecal egg count when grazing sulla (condensed tannin ~10-12%) compared with those grazing lucerne (condensed tannin-free) (Niezen *et al.*, 1995). Similarly, Robertson *et al.* (1995) reported differences in liveweight gain of parasite-infected sheep grazing different forages. Parasite-infection reduced liveweight gain by approximately 37% for sheep grazing condensed tannin forages (sulla or *L. pedunculatus*), those grazing condensed tannin-free forages (lucerne or ryegrass/white clover) had reductions of liveweight gain of approximately 94%. In sheep fed forages containing condensed tannins decreases in faecal egg counts of 25-50% and up to 2.5-fold decrease in worm burdens have been recorded (Niezen *et al.*, 1995; Robertson *et al.* 1995). The improved performance of parasite-infected lambs ingesting condensed tannins may be due to the protein-binding capacities of the tannin or to a direct anthelmintic effect of the condensed tannin (or some other component in that diet) on nematode viability (Niezen *et al.* 1995). As indicated above, the capacity of condensed tannins to bind with protein could enable soluble protein to bypass the rumen, increasing duodenal protein supply once the complex dissociates in the acidic conditions of the abomasum (Martin & Martin 1983; Martin *et al.* 1985). Additional protein supply in parasite-infected animals has been shown to help the host to expel established worm burdens from the GI tract more rapidly and build up immunity to subsequent incoming infective larvae (see section 1.2.3.2).



Dawson *et al.* (1999) reported some of the findings from a research project carried out at the University of Nottingham between 1990 and 1993 evaluating whether the type and quantities of polyphenolics likely to be ingested in the tropics and subtropics have negative effects on absorption and metabolism by gut tissues. Quebracho tannin was shown to survive passage through the rumen and was observed to have been taken up by Peyer's patches in the small intestine of sheep (Dawson *et al.*, 1999). These patches are involved in the stimulation of the immune system, leading to the secretion of IgA into the gut lumen (Weir & Stewart, 1993). It has been postulated that IgA may provide protective immunity against a number of intestinal nematodes including *N. brasiliensis* and *H. contortus* (Wakelin, 1996). Hence, it was postulated that quebracho tannin could promote an immune response in the host and this could provide greater resistance of the host against infections.

### 1.5.2 Tropical and subtropical environments

In tropical and subtropical areas the numbers of free-living stages of helminth parasites found on pasture follow seasonal fluctuations. The rate of development and the longevity of eggs and larvae vary with temperature, rainfall and humidity in different geo-ecological regions (Tembely, 1998). Ndamukong & Ngone (1996) observed *H. contortus* and *Trichostrongylus* *sp.* eggs moulting to form infective larvae in as little as one week, compared to around three weeks in temperate climates, with the larvae surviving for periods of 7 to 13 weeks during the rainy season in a mid to high altitude area of Cameroon (1500-2000m above sea level). In the cool tropical environment of the highlands of Ethiopia (~3000m above sea level), Tembely (1998) observed infective larvae persisted on the pasture for 2 to 6 weeks during the long rainy season, while eggs failed to develop to the L<sub>3</sub> stage during the dry and short rainy season. Thus the combination of high temperatures and rainfall generally favour the development and survival of parasites. Thus, animals in these areas of the world are generally exposed to greater numbers of infective larvae compared to those in more temperate climates (Waller, 1997).

Farmers face a continual battle to control ruminant parasites, attempting to prevent clinical disease that can result in animal mortality and reduce production losses. The use of anthelmintic drugs to reduce worm burdens in such areas is more problematic than in temperate regions. One extreme exists where these drugs are either unaffordable or of such inferior quality that they are not used, while at the other end, continual administration of anthelmintics has developed a situation where total chemotherapy failure to control parasitic infections is becoming reality (Waller, 1997). The need for alternative methods to control internal parasites is paramount in these regions.

Livestock production systems in the tropical and subtropical regions of the world face an ever-increasing demand for products to cope with the escalating human population and the need to improve standards of human nutrition (Waller, 1997). Land for animal production is being lost due to the pressure for human housing and cropping leading to intensification and a heavier reliance on anthelmintics to control internal parasites (Waller, 1997). Available feedstuffs for livestock are often of low quality and fibrous leading to poor rumen function and productivity. Feed supplementation is often not economically feasible, although low-cost minerals and non-protein nitrogen supplements can improve rumen function, increasing feed intake and microbial protein production resulting in increased productivity (Waller, 1999). Improved nutritional status can increase the animals' ability to withstand the effects of parasitism (see section 1.2.3). During the dry seasons the quantity and quality of natural pasture deteriorates. Browse species (shrubs and tree foliage) maintain their nutritive value (often high crude protein content) and greenness during the dry season and can be used to supplement diets (Tolera *et al.*, 1997). However, most browse species contain phenolic compounds which can have both beneficial and negative effects on ruminant production (Mueller-Harvey & Hartley, 1987; Reed *et al.*, 1990; Tolera *et al.*, 1997). One beneficial aspect of feeding condensed tannin-containing forages is the associated reduction in parasitic infections. The use of browse species as fodder could prove to be very important, not only to increase animal liveweight gain but also to reduce parasitic infection. The protection of animals against parasitic infection using locally available indigenous plants would reduce the reliance of the livestock industry on anthelmintic drenches. This is of



particular importance where widespread parasite resistance exists and where the use of anthelmintics is economically impossible.

## 1.6 AIMS OF THE THESIS

The aim of this thesis was to investigate the effects of the dietary inclusion of a model condensed tannin on the parasite burden of animals and the effect of high and low dietary protein supply to the host. The model condensed tannin chosen for the project was quebracho tannin. Previous work at the University of Nottingham has shown that quebracho tannin survives passage through the rumen. Other workers have shown that quebracho tannin is not extensively metabolised by rumen microorganisms (Makkar *et al*, 1995b) and substantial quantities (60-100%) of orally administered quebracho tannin can be recovered in the faeces of ruminant animals (Robbins *et al.*, 1991). In addition quebracho tannin is readily available in large quantities due to its use in the leather industry.

The first objective of the project was to determine whether dietary quebracho tannin reduced parasite burden. This was initially carried out using the *N. brasiliensis*-rat model. Sheep studies using the nematode *T. colubriformis* were then conducted to ensure the findings from rat trials were not confined to the *N. brasiliensis*-rat model. The objective was then to determine the effect of high and low dietary protein concentrations on parasite burdens, and the interaction of the inclusion of quebracho tannin in both diets. This again was carried out using the *N. brasiliensis*-rat model before undertaking studies using the *T. colubriformis*-sheep model. The final objective of the project was to attempt to establish the mechanism through which quebracho tannin was reducing parasitic burden. Rat trials were carried out comparing the effect of dietary quebracho tannin in the immune-suppressed host and also using species of GI nematodes, which either live within the mucosa or are present only in the lumen of the small intestine. Direct toxicity of quebracho tannin against adult nematodes was assessed by monitoring the survival of the worm *in vitro* in a medium containing varying concentrations of quebracho tannin.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 INTRODUCTION

This chapter describes the routine materials and methods used throughout the project. Further details and methods relating to specific pieces of work are described in the relevant experimental chapters. Details of solutions used in the following methods are outlined in the appendix.

#### 2.2 MATERIALS

##### 2.2.1 Animals

###### 2.2.1.1 Rats

Male Wistar rats (80-100 g) were obtained from B & K Universal Ltd., Hull, U.K. Animals were housed in wire metabolism cages [MSD, Modular Systems and Developments Company Ltd., London] in a humidified, temperature-controlled room ( $50 \pm 5\%$  relative humidity,  $20 \pm 1^{\circ}\text{C}$ ) on a 12h light, 12h dark lighting pattern, unless otherwise stated. Feed and water were available *ad libitum* at all times.

###### 2.2.1.2 Sheep

Male and female lambs were raised in-house from birth or purchased from the University of Nottingham farm at the age of weaning (approximately 8 weeks of age) and housed under Home Office regulations. Free access to water and mineral licks was available at all times



### 2.2.2 Reagents

All chemicals were of analytical grade (where available) and were purchased from Sigma Chemical Company (Poole, Dorset), BDH Laboratory Supplies (Poole, Dorset), Merck Ltd. (Lutterworth, Leicestershire) or Fisher Scientific (Loughborough, Leicestershire) unless otherwise stated.

Two sources of quebracho tannin have been used in this thesis. The predominant quebracho tannin used is that which is commercially available through its use in the leather industry. This quebracho extract is an organic material of vegetable origin that contains tannins of the condensed type. It consists largely of polyphenolic polymers associated with a small quantity of carbohydrates. The polyphenols are present mainly as trimers and tetramers formed from a basic monomer (C<sub>15</sub>) consisting of resorcinol or phloroglucinol and catechol or pyrogallol nuclei linked through a central heterocyclic or pyran unit. The commercial quebracho tannin used is cold-soluble (ATO; Hodgesons Chemicals Ltd, Beverley, Hull) a solubilised bisulphited form of the warm-soluble quebracho (ordinary quebracho tannin), a natural extract from the heartwood of the quebracho tree. Chemical treatment of ordinary quebracho tannin with metabisulphite, oxalic acid and EDTA improves the colour and solubility for use in the leather industry. In addition, the quebracho tannin is also spray-dried giving a water content of around 8%. A small quantity of natural quebracho tannin, which was untreated, was also available for use (gift from Ariel Lopez Malto, UNITAN SAICA, Argentina). The condensed tannin content of these two quebracho tannins was evaluated using the method of Terrill *et al.* (1992b). The condensed tannin content of the untreated quebracho tannin was 80% of the content of the commercially available, extracted quebracho tannin.

## 2.3 PARASITOLOGICAL TECHNIQUES

### 2.3.1 Infective larvae

*Nippostrongylus brasiliensis* was maintained by passage through rats in the Division of Behaviour, Infection and Immunity, School of Biological Sciences, University of Nottingham. Infective larvae cultured from faecal material were suspended in phosphate buffered saline (PBS; see appendix) and subcutaneously injected into the host rat.

*Trichinella spiralis* infective larvae from infected stock mice were also supplied by the Division of Behaviour, Infection and Immunity, School of Biological Sciences, University of Nottingham. Infective larvae were suspended in 0.2% agar and orally administered to the host rat using the gavage method.

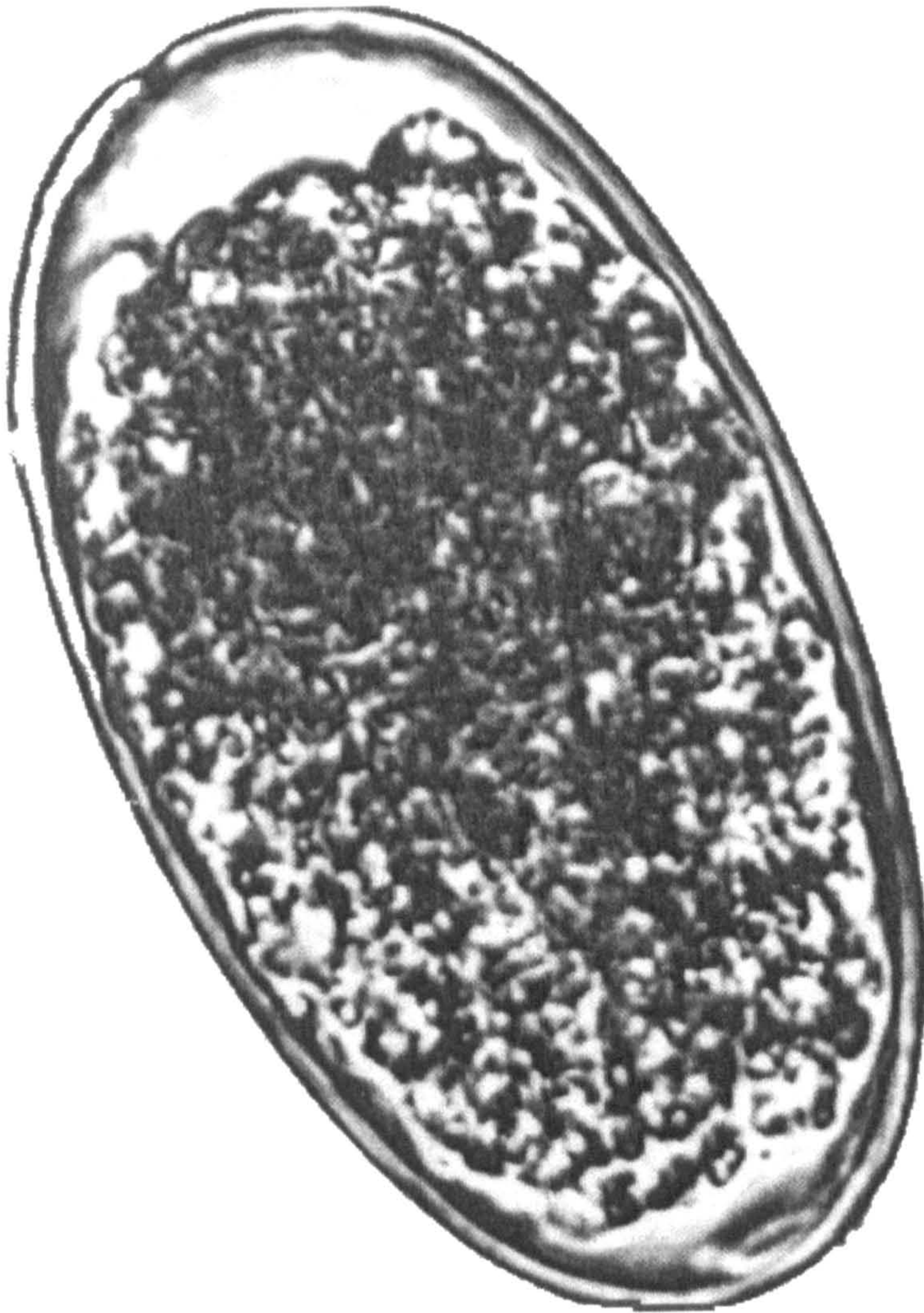
*Trichostrongylus colubriformis* infective larvae were kindly supplied by the Moredun Research Institute, Edinburgh. Batches of infective larvae were sent by overnight courier and stored at 4<sup>0</sup>C before use. The ovine host was orally infected with larvae suspended in distilled water.

### 2.3.2 Faecal egg counts

The number of eggs per gram of faeces (EPG) was determined by the flotation method of Christie and Jackson (1982). *Nippostrongylus* and *Trichostrongylus* are both trichostrongyloid nematodes that reproduce by liberating thin-shelled eggs, a typical trichostrongyloid egg is shown in Figure 2.1.



**Figure 2.1** Typical trichostrongyloid egg



Taken from Urquhart *et al.* (1987)



### *2.3.2.1 Rats*

Faecal material was removed from the litter tray approximately 12h prior to the collection of faeces for egg counting. A known weight of fresh faeces (0.6-1.0g) was collected from the litter tray, transferred to Bijou bottles and soaked in 5 ml distilled water. Samples left to soak for longer than 2h were placed in the cold room (4°C) to delay embryonation. The faecal pellets were disrupted by vortexing followed by mixing with a magnetic follower to ensure the samples were emulsified. The contents of the bottle were passed through a tea strainer into labelled collapsible cellulose acetate tubes [14 x 95 mm, Beckman, California, USA] to remove large faecal debris. A further 5 ml distilled water was added to the bottle and then passed through the strainer to wash any remaining eggs into the tube. The suspension was centrifuged for 3 min at 450 xg. The supernatant was discarded and the pellet resuspended in saturated salt solution and recentrifuged (450 xg for 3 min). Following centrifugation the top fraction, containing the eggs, was separated by clamping the tube with haemostat forceps just below the meniscus. The meniscus solution was then transferred into a labelled 4 ml plastic cuvette. Approximately 2 ml of saturated salt solution was added to the top fraction of the tube (still clamped with haemostat forceps) and added to the cuvette to wash any eggs adhering to the tube surface into the cuvette. The cuvettes were carefully inverted three times and then filled to give a positive meniscus with saturated salt solution. Cuvette caps [Scientific Laboratory Supplies Ltd, Nottingham] were then gently added, excluding air bubbles and the cuvette carefully placed horizontally for at least 15 min to allow the eggs to float to the upper surface. The eggs recovered were counted using a binocular microscope (x 100). A Miller square graticule [Graticules Ltd., Tunbridge, Kent] was used in the eye piece of the microscope. The number of eggs viewed through the graticule square during three traverses of the cuvette surface were counted. The EPG figure was calculated by dividing the number of eggs counted (adjusted for the use with the graticule, see equation 2.1) by the weight of the fresh faeces from which eggs were extracted. EPG figures were then multiplied by total daily faecal output (g) to give total eggs excreted during the 24h period.



**Cuvette dimensions:  $41.5 \times 10 = 415\text{mm}^2$**

For each sample three longitudinal traverses counted:

**Large Miller square:  $1.5 \times 1.5\text{mm}$**

Area counted for each traverse:  $41.5 \times 1.5 = 62.25\text{mm}^2$

Total area counted:  $62.25 \times 3 = 186.75\text{mm}^2$

**Adjustment factor for number of eggs in cuvette:  $415 / 186.75 = 2.22$**

**Small Miller square:  $0.5 \times 0.5\text{mm}$**

Area counted for each traverse:  $41.5 \times 0.5 = 20.75 \text{ mm}^2$

Total area counted:  $20.75 \times 3 = 62.25 \text{ mm}^2$

**Adjustment factor for number of eggs in cuvette:  $415 / 62.25 = 6.67$**

**Equation 2.1** Adjustment factors for number of eggs present in the cuvette when using the large and small Miller square graticule.

#### **2.3.2.2 Sheep**

The method for faecal egg counting was slightly modified from that used for rat faeces (section 2.3.2.1). Faecal samples were collected directly from the rectum. A known weight of faeces (approximately 3 g) were placed in 75 ml plastic container, distilled water was added (10 ml per gram faeces) and mixed to form an emulsion. Exactly 10 ml of the emulsion (equivalent to 1 g of faecal material) was passed through a tea strainer into a labelled collapsible cellulose acetate tube [14 x 95 mm, Beckman, California, USA], 2 ml of distilled water was added to wash any

remaining eggs through the sieve. The suspension was centrifuged, resuspended, centrifuged and counted as above (section 2.3.2.1).

### **2.3.3 Worm recovery**

#### **2.3.3.1 *Rats***

Rats were killed by cervical dislocation and the entire small intestine rapidly removed. The small intestine was opened longitudinally, placed in a net bag, and incubated at 39<sup>0</sup>C for 2h in Hanks' balanced salt solution (HBSS; see appendix) or until no worms were seen attached to the gut. The intestine was discarded and HBSS decanted to leave the extracted worms in approximately 10 ml HBSS. The worms were preserved by adding approximately 10 ml of 10% (v/v) neutral buffered formalin (see appendix), worms were counted using a low powered dissecting microscope.

#### **2.3.3.2 *Sheep***

The sheep were humanely killed by conventional slaughterhouse techniques (stunned and exsanguinated). The entire intestine was removed following ligation of the abomasal/duodenal and ileal/caecal junctions. The small intestine was detached from the mesentery and placed into a large labelled container (10 l) holding approximately 4.5 l of warm saline (0.85% (w/w) sodium chloride at approximately 37<sup>0</sup>C). The entire contents of the small intestine were emptied into the container by running the intestine between the thumb and index finger. The empty intestine was opened longitudinally by running blunt ended scissors through the entire length. The container was then closed and the intestine incubated at 37<sup>0</sup>C with regular agitation for at least 4h. Following incubation the mucosal layer together with any adhering worms were separated from the remaining intestine tissue by running the intestine through the thumb and index finger. The remaining intestinal tissue was discarded. The contents of the container, the digesta together with the mucosal tissue and worms were made up to 5 l with additional 0.85% (w/v) sodium chloride. Following



thorough mixing of the container contents, two 250 ml aliquots were removed and pooled together to give a 10% aliquot. A second 10% aliquot was also stored. To each 500 ml subsample, 25 ml of formaldehyde (to give a 5% (v/v) solution) was added to preserve the worms.

The worms were counted in 50 ml subsamples from the 500 ml aliquot. To each 50ml subsample, 0.5 ml of helminthological iodine (see appendix) was added. The sample was left to stain for approximately 5 min before being examined with a low powered dissecting microscope.

#### **2.3.4 Eggs *in utero***

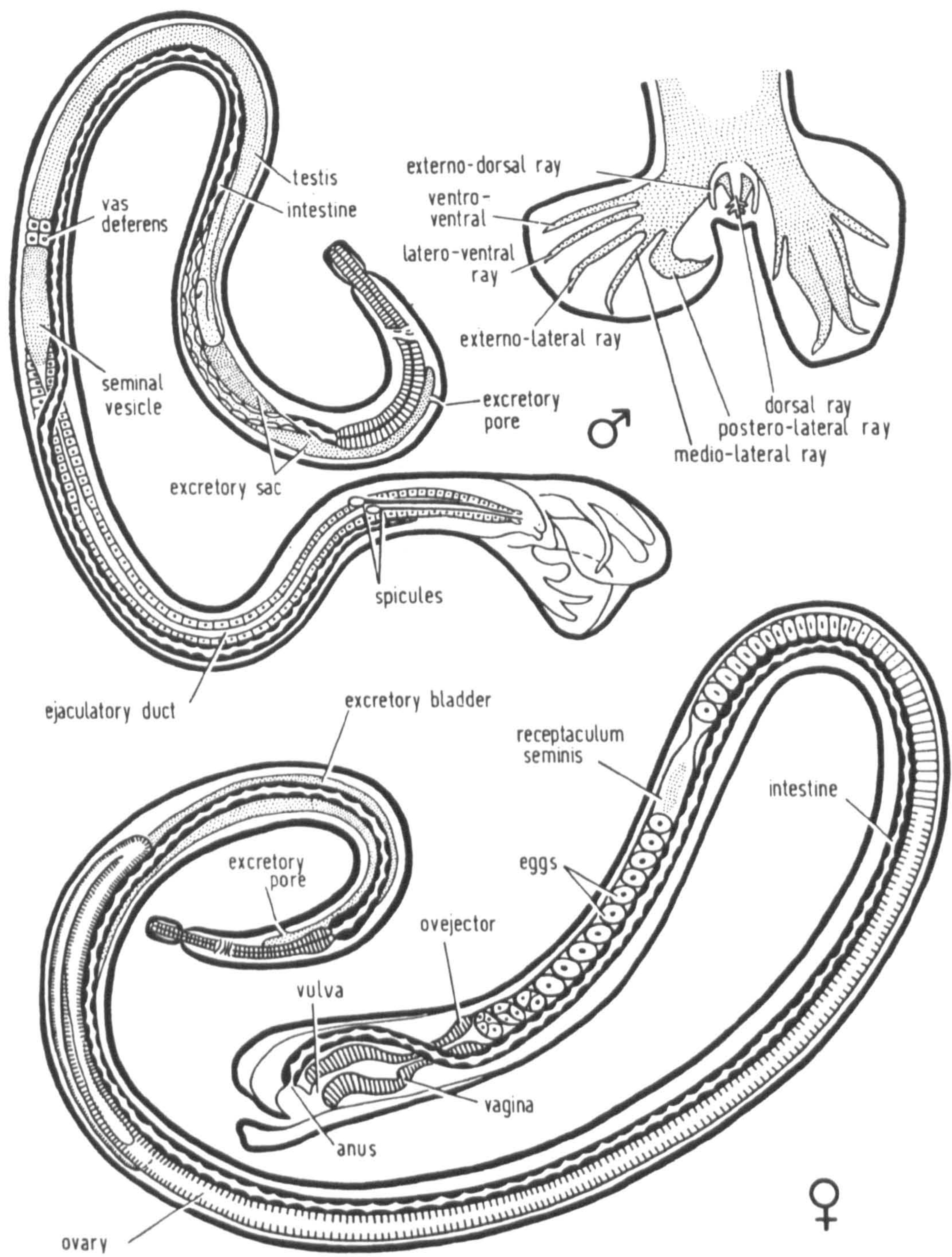
Worms were sexed by identifying the female worms as being the larger and lacking the characteristic bursa forming an umbrella-like expansion surrounding the cloaca of the male (Figure 2.2). In addition, ellipsoidal eggs can be observed within the uterus of the female. The number of eggs visible *in utero* was recorded from a consistent number of randomly chosen from each animal.

#### **2.3.5 Worm motility**

Adult *N. brasiliensis* worms were extracted from the small intestine as above (section 2.3.3.1). Groups of approximately 20 worms (mixed sexes) were placed into petri dishes [35 x 10 mm, Falcon, Becton Dickinson Labware, New Jersey] containing 2 ml HBSS with different concentrations of quebracho tannin ranging from 0% (control dish) through to 4% (w/v) quebracho tannin. The dishes were maintained at 37-38°C on a heating block [Techne Dri-Block D8-3, Cambridge]. The activity of the worms was monitored at various time intervals over a 10h period. Worms were classed as either active or inactive. Inactive worms were defined as



Figure 2.2 *Nippostrongylus brasiliensis*: anatomy of male and female



Taken from Smyth, 1994



those that showed no motility and did not respond to a mechanical stimulus (gentle prodding and lifting of the worms with a mounted needle).

## **2.4 HAEMATOLOGICAL TECHNIQUES**

### **2.4.1 Plasma and serum collection (Sheep)**

Plasma was isolated from 10 ml of peripheral blood obtained by jugular venipuncture following the morning feed, into tubes containing heparin [2500 iu/ml Multiparvin, CP Pharmaceuticals, Wrexham]. The tubes were gently mixed by inversion several times and kept on ice prior to centrifugation at 1700 xg for 15 min. The plasma was carefully separated and stored in two aliquots at -40°C until required. Aliquots of heparinised whole blood (100 µl) were removed prior to centrifugation and retained on ice for eosinophil counting.

Serum was obtained from whole blood (10 ml) collected into untreated tubes and maintained at 4°C overnight prior to centrifugation (1700 xg for 15 min). The serum was carefully separated and stored at -40°C until analysed.

Before analysis, all samples were thawed at room temperature, vortexed and centrifuged at 1700 xg for 15 min.

### **2.4.2 Eosinophil concentrations**

Concentrations of peripheral circulating eosinophils were calculated using the method of Dawkins *et al.* (1989). An aliquot (100 µl) of freshly obtained heparinised blood (section 2.4.1) was added to 900 µl Carpentiers eosinophil counting solution

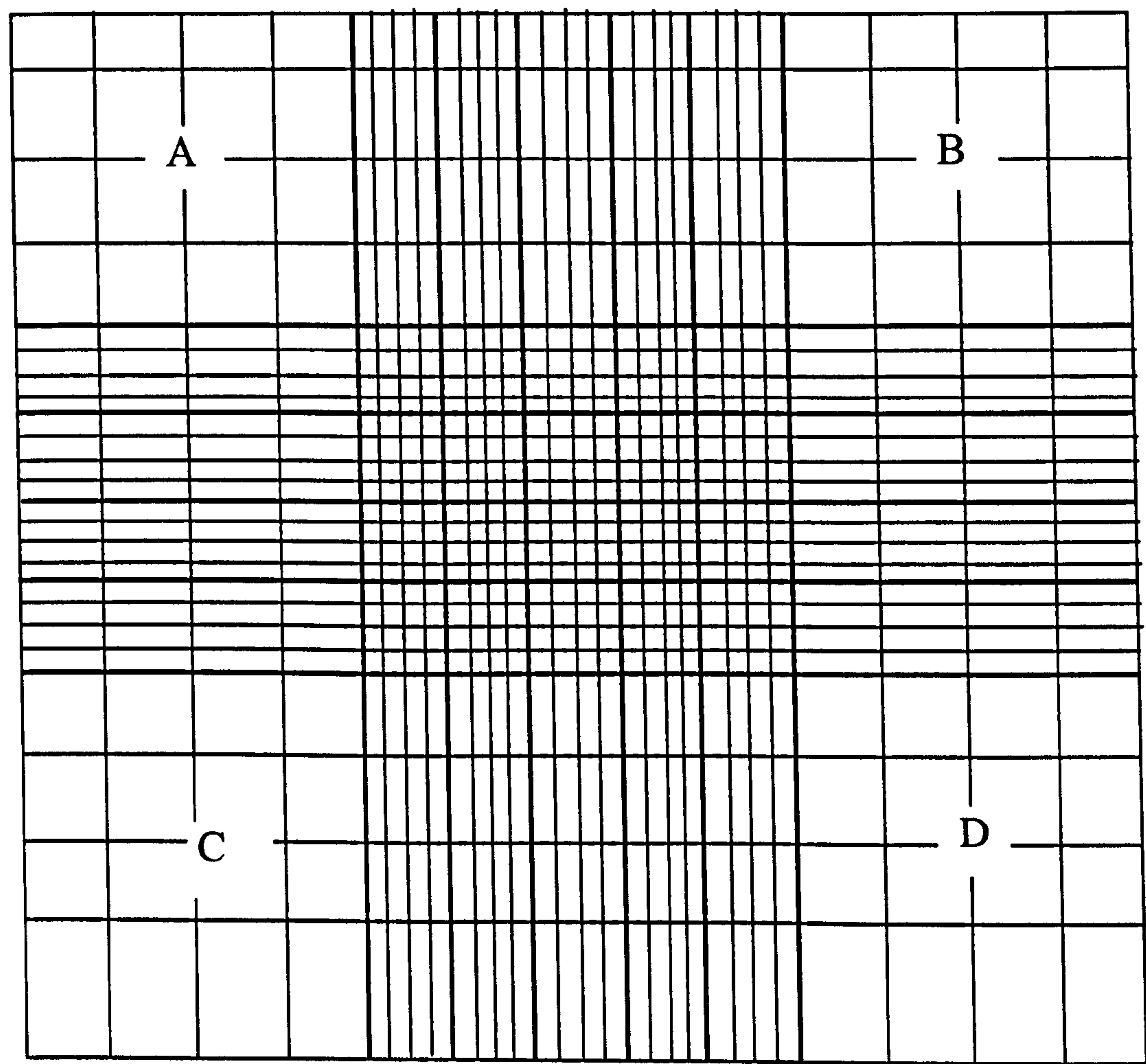
(see appendix) and mixed. The number of eosinophils circulating in the peripheral blood was counted using a haemocytometer [Improved Neubauer Haemocytometer, Hawksey, U.K.] and expressed as the number of cells  $\times 10^4/\text{ml}$  blood. The number of eosinophils present were counted in the appropriate area of the chamber (Figure 2.3) where  $1 \times 10^{-3} \text{ mm}^3$  blood filled one counting area (one cell/ $10 \text{ mm}^{-3}$  equals  $1 \times 10^4/\text{ml}$ ). The haemocytometer was refilled eight times for each sample (4 counting areas per loading of the haemocytometer) unless the counts exceeded 50. Eosinophils were identified as those cells having a lobed nucleus stained pinkish-red (Figure 2.4). The counts were made from blood samples taken at the same time of day for each time point to reduce diurnal variation in eosinophil number.

### 2.4.3 Amino acid concentration

Plasma samples (see section 2.4.1) were deproteinised using sodium salicylic acid. To appropriately labelled eppendorfs [1.5 ml, Sarstedt, Germany] 50 mg sodium salicylic acid was added. One millilitre of plasma and 0.2 ml of 1.0 mM nor-leucine internal standard was added, thoroughly mixed and allowed to stand for 1h on ice. The samples were then microfuged for 5 min at 18000 xg [A13, Jouan; Saint Nazaire, France]. The supernatant was decanted off and the pH adjusted to approximately 2.2 using 4 M lithium hydroxide. Samples were then either immediately loaded onto the amino acid analyser or frozen ( $-40^\circ\text{C}$ ) until required. Samples (100  $\mu\text{l}$ ) were injected into capsules and loaded, with a 0.2 M lithium citrate loading buffer (pH 2.2), onto a cation exchange amino acid analyser [Pharmacia Biochrom 20, Albans, Herts]. A molarity change was created in a resin packed column [25 cm x 4.6 cm] using five lithium citrate cation buffers ranging from 0.2M to 1.65M, each with specific pH (2.80-3.55) producing an increasing pH gradient. The separation of the amino acids by cation exchange was produced by the change in pH and temperature from  $30^\circ\text{C}$  to  $85^\circ\text{C}$  in the column. A buffered ninhydrin solution reacted with the amino group to form a coloured amino acid complex on incubation in a reaction coil at  $135^\circ\text{C}$ . All amino acid complexes were quantified using a

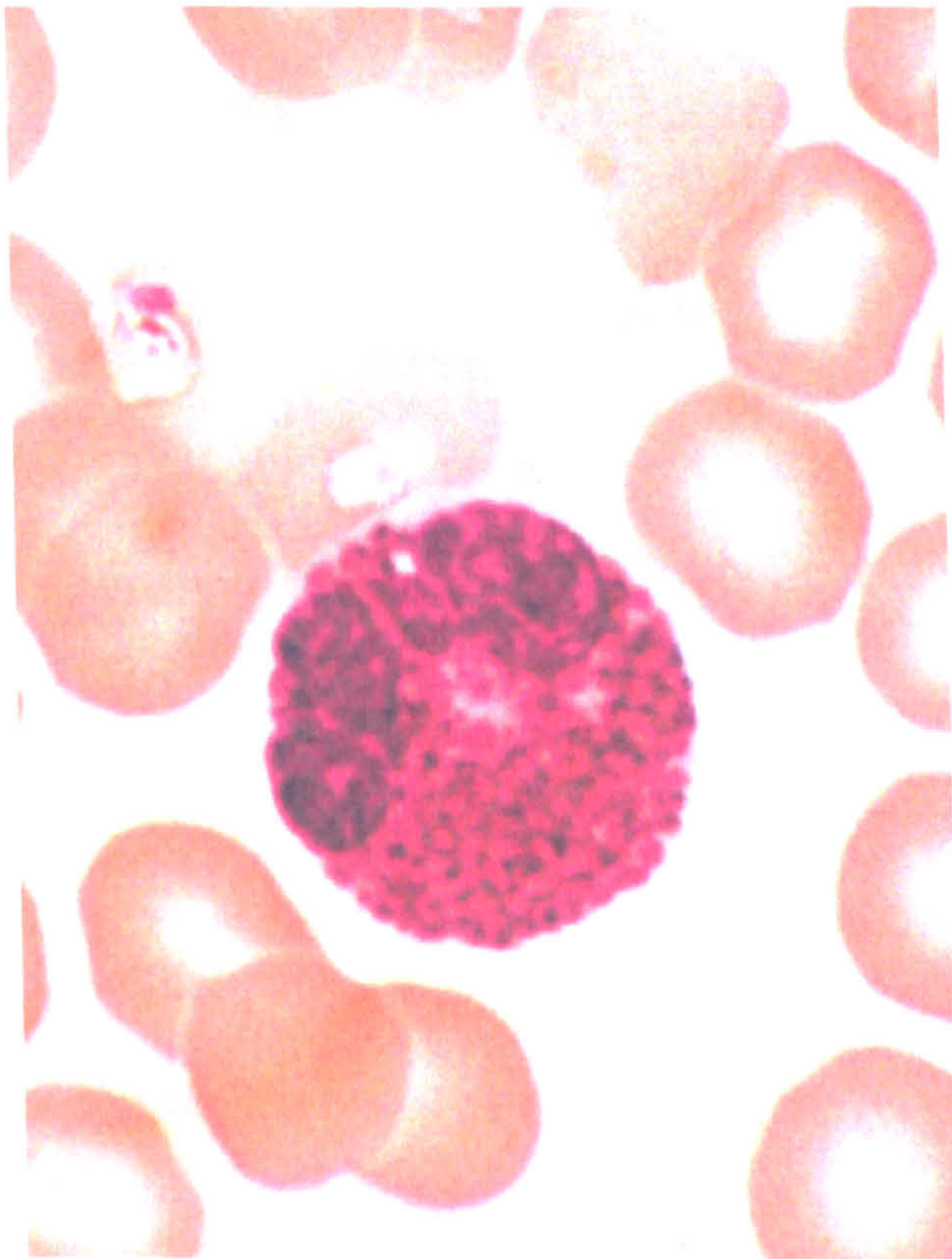


**Figure 2.3** Representation of the area for white blood cell counting using an improved Neubauer counting chamber



White blood cells (eosinophils) were counted in areas A, B, C and D  
(4 of the 9 large counting squares)

**Figure 2.4** Eosinophil (x1600)



Taken from Wheater *et al.* (1987)



colorimeter at 570 nm, except for the proline complex which was measured at 440 nm. Peaks of the individual amino acids were recorded and the area integrated. The concentration of the individual amino acids in each sample was calculated by reference to the peak area of internal standard nor-leucine.

#### **2.4.4 Total protein concentration**

Total protein concentration in ovine plasma (see section 2.4.1) was determined using the method of Burtis and Ashwood (1994). The principle of the method involves the reaction of peptide bonds of proteins with Cu (II) ions in alkaline solutions to form a coloured product whose absorbance is measured spectrophotometrically at 540 nm. The biuret reagent contains sodium potassium tartrate to complex the cupric ions and maintains their solubility in alkaline solution. Iodine was included as an antioxidant. The procedure involved sets of 4 pyrex tubes [28 x 15 mm, Corning, USA] for each standard (C) and unknown sample (U). To the 4 tubes labelled standard (C), tube 1 (blank) contained 5 ml of biuret blank reagent (CB; see appendix), tubes 2, 3 & 4 (test) contained 5 ml biuret reagent (CT; see appendix). To each of the 4 tubes 100 µl of BSA solution (dialysed Fraction V powder, essentially fatty acid free) of known protein content ( $C_C$ , 7.0 g/l) was added and mixed. To the 4 tubes labelled unknown (U), tube 1 (blank) contained 5 ml of biuret blank reagent (UB), tubes 2, 3 & 4 (test) contained 5 ml biuret reagent (UT). To each of the 4 sample tubes 100 µl of sample was added and mixed. In addition, 2 tubes containing 5 ml biuret reagent and 100 µl of distilled water were used as reagent blanks (RB). All tubes were incubated at room temperature for 30 min and the optical density read at 540 nm. Zero absorbance was set using 5 ml biuret blank reagent containing 100µl distilled water.

The protein concentration of the unknown sample was calculated as shown in Equation 2.2.

$$\text{Total protein, g/l} = (A_{UT} - A_{UB} - A_{RB}) / (A_{CT} - A_{UB} - A_{RB}) \times C_C$$

**Equation 2.2**

To ensure the method was linear over the range of values obtained a standard curve was repeated at regular intervals using a concentration range of BSA standards from 5 to 10 g/l. The between-assay CV was less than 2%.

#### **2.4.5 Albumin concentration**

Albumin concentration in ovine plasma (see section 2.3.1) was determined using the method of Burtis and Ashwood (1994). The principle of the method involves the binding of albumin and bromocresol green at pH 4.2 where albumin acts as the cation to bind the anionic dye. The absorbance of the bromocresol green-albumin complex was determined spectrophotometrically at 628 nm. Experimentally, the spectrophotometer was set to zero absorbance at 628 nm with water. The absorbance of bromocresol green reagent (see appendix) was read and the zero absorbance reset. To tubes containing 5 ml bromocresol green reagent, 20 µl of appropriate unknown sample (U) or standard (C; 3.5 g/l BSA) was added, the solution mixed and the optical density read at 30 (±3) sec after the addition.

The albumin concentration of the unknown sample was calculated as shown in Equation 2.3

$$\text{Albumin, g/l} = (A_U / A_C) \times C_C$$

**Equation 2.3**



To ensure the method was linear over the range of values obtained a standard curve was repeated at regular intervals using appropriately diluted solutions of BSA (dialysed Fraction V powder, essentially fatty acid free). BSA standards were used over the concentration range of 1 to 5 g/l. A between-assay CV of less than 4% and within-assay CV of less than 3% was obtained.

#### **2.4.6 Globulin concentration**

The globulin concentration in plasma was estimated by the difference between total protein and albumin concentration

### **2.5 IMMUNOLOGICAL TECHNIQUES**

#### **2.5.1 Enzyme linked immunosorbant assay (ELISA)**

The principle behind an indirect ELISA is the coating of a solid phase with antigen to which serum antibodies bind. An anti-immunoglobulin-enzyme conjugate is allowed to react with the serum antibody. A colour substrate is then added which binds to the enzyme-conjugate enabling the reaction to be measured colorimetrically.

The method of the ELISA used in this study to measure the antibody response to ovalbumin was as follows:

Flexible 96-well microtitre assay plates [Falcon 3912 Microtest III flexible assay plates] were used as the solid support. Plates were coated with 200  $\mu$ l of 5  $\mu$ g/ml solution of antigen [Albumin, chicken egg, Grade V] prepared in carbonate-bicarbonate buffer (pH 9.6; see appendix) per well. The plates were wrapped in cling film and incubated overnight at 4°C to allow the antigen to absorb onto the solid

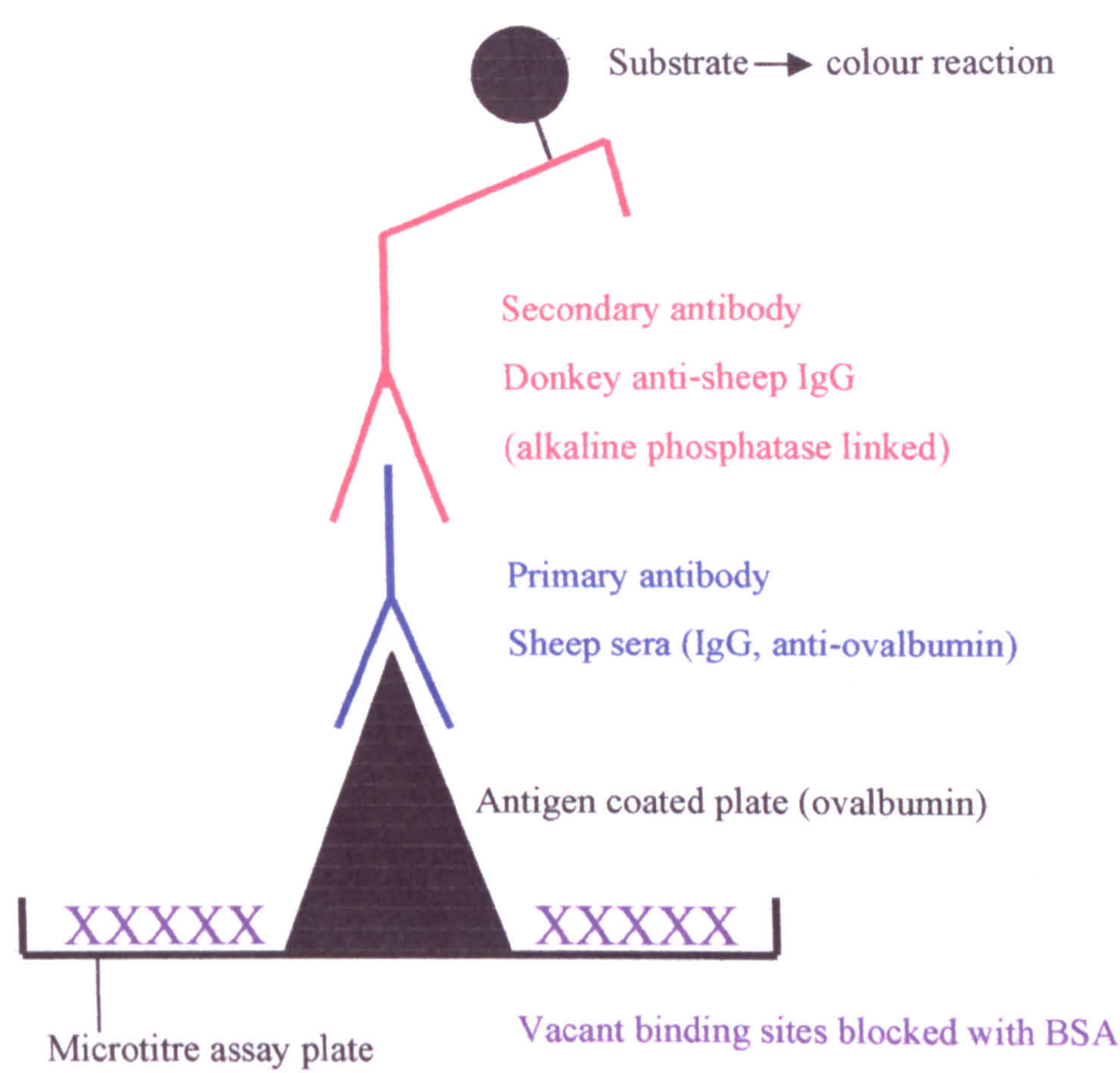
surface of the wells. The coating solution was removed from the plates by washing quickly three times with PBS containing 0.05% (v/v) Tween 20 detergent. Any vacant sites on the solid phase were blocked with BSA. One hundred microlitres 3% (w/v) BSA [dialysed Fraction V powder, essentially fatty acid free] in 0.05% (v/v) PBS-Tween 20 was added to each well, the plates were wrapped in cling film and incubated at room temperature for 60 min, then washed quickly three times with 0.05% (v/v) PBS-Tween 20 to remove the excess blocking solution. Serum containing antibodies to ovalbumin were then added to the plates Fifty microlitres of a 1/1600 dilution of sera in 0.05 % (v/v) PBS-Tween 20 was added to the plate wells in triplicate and incubated at room temperature for 90 min, before being washed as before. A secondary antibody, donkey anti-sheep IgG (whole molecule) conjugated to alkaline phosphatase suspended in 0.05% (v/v) PBS-Tween 20 was diluted to 1:2000 and added to the plate (50 µl/well) and incubated for 90 min at room temperature. The plate was then washed as before followed by two longer washes where 0.05% (v/v) PBS-Tween 20 was held in the wells for 3 min before emptying to ensure all unbound secondary antibody was removed. The colour reaction was developed by adding 100 µl of alkaline phosphatase substrate (1 tablet of p-nitrophenyl phosphate, and 1 tablet of Tris buffer dissolved in 5 ml of distilled water) to each well. The plate was incubated in the dark at room temperature until a suitable colour intensity was attained. The resulting colour reaction was measured at 410 nm using a Dynatech MR5000 multiwell plate reader. The reaction is schematically shown in Figure 2.5. A standard positive serum sample (an infected lamb) was included in each ELISA plate and the results adjusted to that sample having an optical density of 1.0. A negative sample using serum from an uninfected animal prior to ovalbumin injection was also included on all plates.

## 2.6 NITROGEN AND DRY MATTER ANALYSIS

The nitrogen content of feed, urine and faecal samples were determined by Kjeldahl analysis.



**Figure 2.5** Schematic diagram summarising the reaction involved using ELISA





### 2.6.1 Sample collection and preparation

Daily faecal and urine collections were collected from rats housed in metabolism bowls [Techniplast metabolic cages, Biotech, Northants] for a period of 12 days. Total urine volume was measured and frozen at  $-20^{\circ}\text{C}$  until analysed for nitrogen content. Total faecal output was recorded, freeze-dried to constant weight and finely ground using a pestle and mortar. A known amount (approximately 0.5 g) of freeze-dried faecal material was accurately weighed into crucibles and placed in a  $100^{\circ}\text{C}$  drying oven overnight (16h) and reweighed to establish the dry matter content of the sample (Equation 2.4). Samples which were not analysed for nitrogen content were dried to constant weight in a  $70^{\circ}\text{C}$  vacuum oven [Gallenkamp, UK] (Equation 2.5).

### 2.6.2 Digestion

Dry samples of feed and faecal material (approximately 0.5 g; accurate to four decimal places) were weighed into nitrogen-free filter paper [Whatman No. 1, 90mm], which was folded to prevent loss of sample before nitrogen analysis. Urine samples were thawed, centrifuged at 192 xg for 5 min to sediment any particulate material. Aliquots (3.0 ml) were then removed for nitrogen analysis. The samples (feed, faecal and urine) were placed into individual Kjeldahl tubes with one catalytic Kjeltab CX (each tablet containing 5 g  $\text{K}_2\text{SO}_4$  and 0.5 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) [Thompson & Capper Ltd., Runcorn, Cheshire] and 10ml concentrated sulphuric acid. Digestion occurred by placing the Kjeldahl tubes in a digestion rack on a pre-heated Kjeldatherm heating block at  $400^{\circ}\text{C}$  [Gerhardt Turbosog, Brackley, Northants] situated in a fume cupboard with a manifold connected to a scrubber unit [Gerhardt Turbosog, Brackley, Northants] to remove fumes that evolved. After the samples turned green in colour (approximately 30 min), the digestion was continued for a further 20 min. The samples were then left to cool.



$$\text{Freeze dried weight} = \frac{((\text{fresh faeces} + \text{container}) - \text{container})}{((\text{freeze dried weight} + \text{container}) - \text{container})}$$

$$\text{Dry weight} = \frac{((\text{freeze dried weight} + \text{container}) - \text{container})}{((\text{dry weight} + \text{container}) - \text{container})}$$

$$\% \text{ dry matter} = (\text{dry weight} / \text{fresh weight}) \times 100$$

**Equation 2.4** Dry matter calculation when samples freeze dried and then dried to constant weight in 100°C oven

$$\text{Dry weight} = \frac{((\text{fresh weight} + \text{container}) - \text{container})}{((\text{dry weight} + \text{container}) - \text{container})}$$

$$\% \text{ dry matter} = (\text{dry weight} / \text{fresh weight}) \times 100$$

**Equation 2.5** Dry matter calculation when samples dried to constant weight in a 70°C vacuum oven

### 2.6.3 Distillation

The samples were subsequently distilled by individually placing the Kjeldahl tubes on a Vapodest 5 distillation apparatus [Gerhardt UK Ltd., Brackley, Northants]. Sodium hydroxide (50ml; 40% (w/v)) and 50 ml distilled water was added to each tube and mixed. The sample was steam-distilled for 4 min into 50 ml cooled saturated boric acid (5% (w/v)), the evolved ammonia was collected in the boric acid and then titrated against 0.2M hydrochloric acid. The end point was determined by pH.

### 2.6.4 Calculation of nitrogen present in the sample

The nitrogen present in the sample was calculated from the volume of hydrochloric acid required to complete the reaction, where 1 l of 1.0M hydrochloric acid reacts with 14 g nitrogen, equivalent to 1 ml 0.2M hydrochloric acid reacting with 0.0028 g nitrogen. The calculation used to obtain percentage nitrogen (N) in the sample is shown in equation 2.6 below:

$$\% \text{ N in sample} = (\text{Titre} \times 0.0028 \times 100) / \text{sample weight (g)}$$

**Equation 2.6**



## 2.7 CHROMIUM ANALYSIS

The chromium content in faecal and feed samples was determined using the method of Siddons *et al.* (1985).

### 2.7.1 Sample preparation

Faecal and feed samples were dried in a 70°C vacuum oven [Gallenkamp, UK] to constant weight. The dry samples were finely ground and passed through a 1mm screen prior to ashing in a muffle furnace [Stuart Scientific Co. Ltd., Redhill, Surrey] at 500°C for 16h in 25 ml pyrex bottles [Bibby Sterilin Ltd., Stone, Staffs.]. The samples were allowed to cool prior to the addition of 6 ml digestion acid (see appendix). A flat plate heater [Chiltern Scientific Instrumentation Ltd., Bucks] was used to heat the solution to boiling and 3 ml potassium bromate solution (45 g/l) added. Heating was continued until the solution turned deep purple in colour and white fumes evolved after which the mixture was heated for a further 3 min and then allowed to cool. The samples were then diluted to 25ml with distilled water and stored at 4°C in 50 ml tubes [Falcon, Becton Dickinson Labware, New Jersey] prior to atomic absorption flame analysis.

### 2.7.2 Atomic absorption spectrometry

The atomic absorption spectrophotometer was set up for use with a nitrous oxide/acetylene flame, using the chromium lamp and the wavelength was set to 357.9nm and a slit width of 0.2. The flame was optimised using 100 ppm chromium solution (25% (v/v) digestion acid) and distilled water. The chromium content of the solutions were measured against a series of standards ranging from 2 to 10 ppm chromium solution (potassium dichromate) prepared in 25 % (v/v) digestion acid.

## 2.8 STATISTICAL ANALYSIS

Details of the statistical analysis are given in each relevant chapter. In all case data were analysed using Genstat 5™ (Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Differences were assumed to be significantly different at  $p \leq 0.05$ . Trends were considered between  $0.10 > p > 0.05$ .



**CHAPTER 3**  
**THE EFFECTS OF DIETARY PROTEIN CONCENTRATION**  
**AND QUEBRACHO TANNIN INCLUSION ON THE**  
**ESTABLISHMENT AND PERSISTENCE OF**  
***NIPPOSTRONGYLUS BRASILIENSIS* IN THE RAT**  
**(RAT TRIALS 1, 2 & 3)**

**3.1 INTRODUCTION**

This chapter describes the initial work carried out using the rat and the small intestinal nematode *Nippostrongylus brasiliensis* as a model for later work involving the sheep and *Trichostrongylus colubriformis*.

Condensed tannins, often found in browse plants in arid and semi-arid regions, are generally regarded as anti-nutritional especially in monogastric animals. This occurs through a reduction in nutrient availability, in particular by forming complexes with proteins (Jansman, 1993). Conversely, the anti-nutritional activity of tannins may not be primarily due to a reduction in nutrient digestibility, but by the inhibition of metabolic events occurring after digestion and absorption of the nutrients (Bernays *et al.*, 1989; Mole *et al.*, 1993). Some mammals have the capability to secrete proline-rich proteins (PRPs) in their saliva and this it thought to convey some protection against dietary tannin activity in the gastrointestinal tract. PRPs have a high affinity for tannins, being 10-fold greater than for bovine serum albumin (Mehansho *et al.*, 1987) thus complexing with dietary tannin in preference to dietary proteins. Animals that have been reported to secrete salivary PRPs include man, rats and browse species such as deer or antelope (Austin *et al.*, 1989). When dietary tannin concentration exceeds the capacity of salivary PRPs the remaining uncomplexed dietary tannin would then form complexes with protein in the diet, reducing dietary protein availability. Therefore feeding a high tannin-containing diet with additional

dietary protein may be able to reduce some deleterious effects of dietary tannin in the diet. To establish this, the current trials were carried out feeding either a high or low protein diet with the inclusion of 40 g quebracho tannin/kg.

Many reviews and papers have been published reporting the effect of small intestinal parasitism on protein digestion and metabolism in the sheep (Poppi *et al.*, 1986, 1990; Parkins & Holmes, 1989), but also in other hosts including the rat (Cummins *et al.*, 1978, 1987; Frandsen, 1985) The outcome of these is that the infected animal has a greater requirement for dietary protein. Increasing the protein supply to the lower gastrointestinal tract can help overcome the debilitating effects of parasitism and increase resistance of the host to re-infection. The effect of condensed tannin on small intestinal parasitic infection has not been fully established. The data, which is available, is limited to sheep grazing tannin-containing forages. Work published by Niezen *et al.* (1995, 1996) where natural forage containing low concentrations of condensed tannins was grazed by lambs showed that small intestinal nematode infection was reduced. To determine if dietary condensed tannins could provide protection against parasite infection in monogastrics the following trials were conducted.

The three trials reported in this chapter involved the *N. brasiliensis*-rat model. Experimental diets were designed to investigate the effects of dietary protein concentration and condensed tannin inclusion on the establishment and persistence of *N. brasiliensis* in the small intestine. The condensed tannin used in the first instance was the commercially available quebracho tannin. Quebracho, which has been extracted with metabisulphite, oxalic acid and EDTA to improve solubility and colour, for use in the leather industry, and termed extracted quebracho tannin (eQT). Consequently, there is the possibility that any effect of dietary eQT could be from one or more chemical residues present in the compound due to the extraction process. A small quantity of untreated quebracho tannin (uQT) was available. High and low protein diets were also made replacing eQT with uQT in an attempt to clarify if the effect of eQT was due to the presence of quebracho tannin or chemical residues in the compound.



## 3.2 MATERIALS AND METHODS

### 3.2.1 Experimental diets

The experimental diets used in the following rat trials are shown in Table 3.1. Diets were formulated to be either high protein (H) or low protein (L), with casein as the sole protein source. National Research Council (1995) figures estimate that the protein requirement for maximal growth of the rat is 12 percent when highly digestible protein of balanced amino acid pattern is fed. Diet H contained 250g casein/kg, diet L contained 100g casein/kg. The nitrogen content of casein was determined by the Kjeldahl method (section 2.6). The protein concentration ( $N \times 6.25$ ) of casein was calculated to be 81% (w/w), giving a protein concentration of approximately 20% (w/w) and 8% (w/w) for diets H and L respectively, these being greater and less than the figure of 12 percent quoted to support maximal growth. To account for the additional casein present in diet H the quantity of maize starch was reduced by 150g/kg. The control diets (H & L) contained 80g alphacel/kg (ICN, USA) a cellulose compound with no nutritional content. Quebracho tannin was considered to be nutritionally inert. In the diets containing 40 g quebracho tannin/kg, the quantity of alphacel was reduced to 40g/kg to ensure the same nutrient presentation to rats fed the control or tannin diet at the same dietary protein concentration. Quebracho tannin used in the diets was either the extracted tannin (eQT) or untreated tannin (uQT).

### 3.2.2 Trial 1 investigating the effect of high and low dietary protein concentration, with and without eQT, on the establishment of *N. brasiliensis* in the small intestine of rats

#### 3.2.2.1 Animals and diet

Thirty-six male Wistar rats (initial weight  $126.08 \pm 5.57$  g) were given 3 days to

**Table 3.1** Composition of the synthetic diets fed to rats (g/kg)

| Ingredient             | Supplier                      | High protein | High protein + eQT | Low protein | Low protein + eQT |
|------------------------|-------------------------------|--------------|--------------------|-------------|-------------------|
| Maize Starch           | Dickens, Nottingham.          | 269          | 269                | 419         | 419               |
| Casein                 | Bacarel, Essex.               | 250          | 250                | 100         | 100               |
| Glucose                | Dickens, Nottingham.          | 300          | 300                | 300         | 300               |
| Alphacel               | ICN, USA                      | 80           | 40                 | 80          | 40                |
| Mins/Vits <sup>1</sup> | SDS, Witham, Essex.           | 50           | 50                 | 50          | 50                |
| L-methionine           | Sigma, Dorset.                | 1            | 1                  | 1           | 1                 |
| Corn Oil               | Sainsbury's                   | 50           | 50                 | 50          | 50                |
| Quebracho Tannin       | Hodgesons Chemicals Ltd. Hull | 0            | 40                 | 0           | 40                |

Where stated in the text eQT was replaced with untreated quebracho tannin (uQT).

Mins/Vits<sup>1</sup> Complete mineral and vitamin mix prepared for rodents



acclimatise to their new environment before being randomly allocated to two dietary groups. Half of the animals (18 rats) were fed diet H, the remaining 18 were fed diet L. Seven days later, eQT was introduced to half the animals in both dietary protein groups, giving 4 dietary groups of 9 animals (H, H + eQT, L & L + eQT). The rats were individually housed and fed *ad libitum* as described in section 2.2.1.1. Bodyweights and feed intakes were recorded daily [Ohaus GT 4800 electronic balance, New Jersey, USA,].

#### 3.2.2.2 Nematode infection

All animals were maintained on the experimental diets for 16 days after the introduction of eQT, before being subcutaneously injected with 1000 infective (L<sub>3</sub>) *N. brasiliensis* suspended in PBS (section 2.3.1.).

#### 3.2.2.3 Experimental protocol

Six days after the introduction of the eQT diets, all 36 rats (groups 1-4) were transferred into individual metabolism bowls [Techniplast metabolic cages, Biotech, Northants]. Following a 5 day acclimatisation period, daily feed and water intake and faecal and urine excretion was recorded for each animal at 10.00h daily. The collection period commenced 7 days prior to parasite infection and continued until day 5 p.i. Total 24h faecal and urine collection were weighed and frozen (-20°C) on the day of collection. Feed samples were collected daily and stored at -20°C. Faecal samples were freeze dried and then oven dried to constant weight to give dry matter values (section 2.6). Faecal samples were ground using a pestle and mortar before analysed for nitrogen content. Feed samples were thawed before nitrogen analysis. Urine samples were thawed, vortexed and centrifuged at 192 xg for 5 min to remove any precipitation prior to nitrogen analysis (section 2.6).

The rats were killed 5 days p.i. by cervical dislocation. The small intestine was quickly removed and the worm burden recovered (section 2.3.3.1). Five female

worms recovered from each rat were randomly selected and the number of eggs *in utero* counted and recorded (section 2.3.4).

### **3.2.3 Trial 2 investigating the effect of high and low dietary protein concentration, with and without quebracho tannin, on the establishment of *N. brasiliensis* in the small intestine of rats**

#### ***3.2.3.1 Animals and diet***

The protocol followed was based on that of trial 1 (section 3.2.2.1). In addition to the 4 experimental diets used in trial 1, a further two diets (H & L) containing 40g uQT/kg replacing 40 g alphacel/kg (H + uQT & L + uQT) were fed. These diets were included to compare the effects of chemically treated and untreated quebracho tannin. Thirty-six male Wistar rats (initial bodyweight  $142.4 \pm 7.5$  g) were randomly allocated to diet H (18 rats) or diet L (18 rats) for 7 days. Each group was then subdivided into 3 groups of 6 rats and fed either the eQT, uQT or control diet at that dietary protein concentration. The animals were individually housed and fed *ad libitum* as described in section 2.2.1.1. Body weights and feed intakes were recorded 3 times weekly [Ohaus GT 4800 electronic balance, New Jersey, USA].

#### ***3.2.3.2 Nematode infection***

All animals were maintained on the experimental diets for 16 days after the introduction of dietary tannin, before being subcutaneously injected with 2500 L<sub>3</sub> *N. brasiliensis* suspended in PBS (section 2.3.1.).

#### ***3.2.3.3 Experimental protocol***

All rats were killed 5 days p.i. and the worm burden extracted from the small intestine as described in section 2.3.3.1. In addition 20 female worms extracted from



each rat were randomly selected and the number of eggs *in utero* counted and recorded (section 2.3.4).

### **3.2.4 Trial 3 investigating the effect of high and low dietary protein concentration, with and without quebracho tannin on the daily faecal egg output from *N. brasiliensis* infected rats**

#### **3.2.4.1 Animals and diet**

Forty-two male Wistar rats (initial mean bodyweight  $137.6 \pm 8.9$  g) were randomly allocated two groups and fed either diet L (24 rats) or the diet H (18 rats). After 7 days the rats in each dietary protein group were subdivided into groups of 6 rats. Groups were fed eQT, uQT or remained being fed the control diet. The remaining 6 animals initially fed diet L remained on this diet until nematode infection, at which time they were then changed onto the L + eQT diet (L-L + eQT). The rats were individually housed and fed *ad libitum* as described in section 2.2.1.1. Body weights and feed intakes were recorded 3 times a week [Ohaus GT 4800 electronic balance, New Jersey, USA].

#### **3.2.4.2 Nematode infection**

All animals were maintained on the experimental diets for 16 days after the introduction of dietary tannin, before being subcutaneously injected with 2500 L<sub>3</sub> *N. brasiliensis* suspended in PBS (section 2.3.1.).

#### **3.2.3.3 Experimental protocol**

Faecal egg counts were carried out on all rats as described in section 2.3.2.1 between days 5 and 10 post infection (p.i.). Faeces were removed from the faecal tray at 20.00h prior to the egg counting the following day, ensuring the faeces collected at 08.00h were fresh. Total faecal collection was aided by lining the faecal trays with

paper. Total daily faecal egg output was calculated for the period from 20.00h to the following 20.00h. Faecal pellets collected at 20.00h were placed in pre-weighed plastic tubes. The following morning fresh faeces were collected for egg counting (weight noted) and any remaining faecal pellets were added to the previous evening's collection. The tubes plus faecal pellets were weighed and then dried to constant weight in a 70°C vacuum oven. The samples were then reweighed and a dry matter value calculated (section 2.6.1, equation 2.4).

### 3.2.4 Statistical Analysis

#### *3.2.4.1 Trial 1 investigating the effect of high and low dietary protein concentration, with and without eQT, on the establishment of N. brasiliensis in the small intestine of rats*

Data from trial 1 were analysed as a four-treatment completely randomised experiment using one-way analysis of variance [Genstat 5, Release 4.1; Lawes Agricultural Trust, Rothamsted]. Variation between the four treatments was partitioned in a 2 x 2 factorial to compare the effects of dietary protein concentration (high and low) with and without dietary tannin. Daily liveweight gains were calculated by linear regression for each animal, slopes were tested by one-way analysis of variance. Data were blocked for rat and differences were assumed to be significantly different at  $p \leq 0.05$ .

#### *3.2.4.2 Trials 2 & 3 Trial 2 investigating the effect of high and low dietary protein concentration, with and without quebracho tannin, on the establishment of N. brasiliensis in the small intestine and the daily faecal egg output of N. brasiliensis infected*

Data from trials 2 & 3 were analysed as a six-treatment completely randomised experiment using one-way analysis of variance [Genstat 5, Release 4.1; Lawes Agricultural Trust, Rothamsted]. Variation between the six treatments was partitioned to compare the effects of dietary protein concentration (high and low),



with and without dietary tannin and tannin type (eQT and uQT). DLWG were calculated by linear regression for each animal, slopes were tested by one-way analysis of variance. Data were blocked for rat and differences were assumed to be significantly different at  $p \leq 0.05$ .

Repeated measures were used to analyse changes with time within-treatment (faecal egg counts during trial 3). The Greenhouse-Geisser epsilon factor obtained from repeated measures analysis was used to adjust the degrees of freedom for time x diet interactions (Winer *et al.*, 1991). Data were further partitioned into linear, quadratic and cubic trends.

### 3.3 RESULTS

#### 3.3.1 Trial 1 investigating the effect of high and low dietary protein concentration, with and without eQT, on the establishment of *N. brasiliensis* in the small intestine of rats

##### 3.3.1.1 Liveweight and feed intake

The mean growth rates of the animals in trial 1 fed the four dietary treatments are shown in Figure 3.1. Rats fed diet H had the most rapid weight gain compared to the remaining 3 dietary groups. The mean DLWG of the rats (from the introduction of the eQT diets to 5 days p.i.) are shown in Table 3.2. The rats fed diet L had a significantly depressed growth rate compared to those rats fed diet H ( $p < 0.001$ ). The inclusion of eQT in diet H reduced mean growth rate of the rats compared to the H-fed rats ( $p < 0.001$ ) similar to that of the L-fed rats ( $p > 0.2$ ). A significant depression of growth rate was seen by the inclusion of eQT into the L-fed rats ( $p < 0.01$ ).

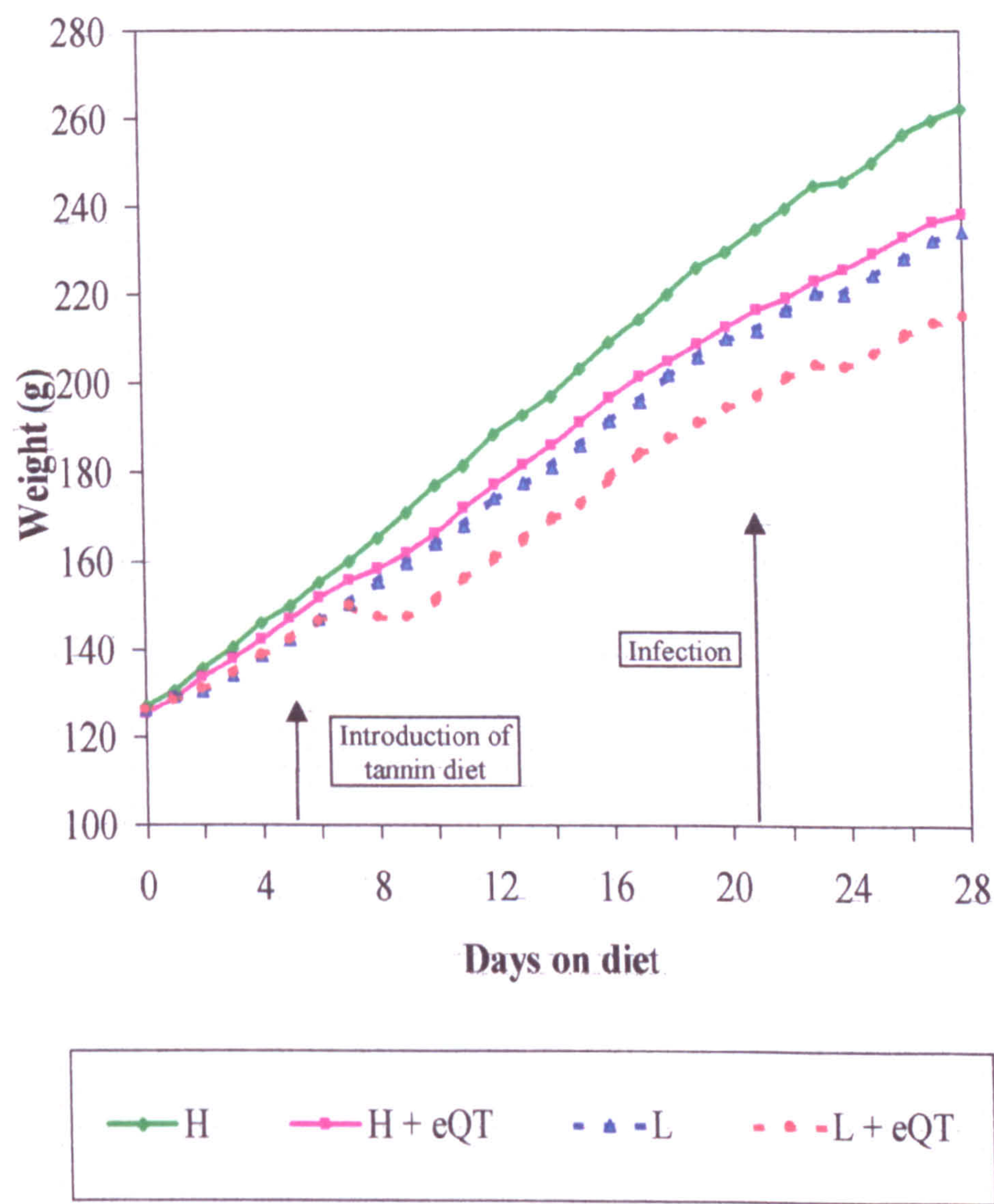
The mean daily feed intakes of the 4 dietary groups, taken from the introduction of eQT diets until 5 days p.i., are also shown in Table 3.2. A non-significant reduction in feed intake was seen by the inclusion of dietary eQT. Rats fed diet L ( $\pm$  eQT) tended to consume more feed than H  $\pm$  eQT-fed rats.

##### 3.3.1.2 Nitrogen balance

The animals were housed individually in metabolism bowls and were given 5 days to acclimatise before total intake and nitrogen excretion were recorded. Data shown in Table 3.3 compares diet treatment (high and low protein, with and without eQT) on nitrogen balance measurements before nematode infection. Table 3.4 contains nitrogen balance measurements recorded p.i.



**Figure 3.1** Effect of high and low dietary protein concentration, with and without eQT, on growth of rats infected with *N. brasiliensis* (Trial 1)



**Table 3.2** Effect of high and low dietary protein concentration, with and without eQT, on growth rate and daily feed intake of rats (Trial 1)

| Diet                 | H                    | H + eQT            | L                  | L + eQT              | s.e.d.<br>(56 d.f.) |
|----------------------|----------------------|--------------------|--------------------|----------------------|---------------------|
| Growth rate<br>(g/d) | 5.04 <sup>a</sup>    | 4.12 <sup>b</sup>  | 4.07 <sup>b</sup>  | 3.50 <sup>c</sup>    | 0.237               |
| Feed intake<br>(g/d) | 19.83 <sup>a,b</sup> | 18.67 <sup>a</sup> | 20.45 <sup>b</sup> | 19.29 <sup>a,b</sup> | 0.706               |

a,b,c means with different superscripts within a row are significantly different  $p<0.05$ .

Results are means of 21 observations from the introduction of dietary eQT to appropriate dietary treatments to 5 days p.i.



**Table 3.3** Effect of high and low dietary protein concentration, with and without eQT, on mean daily nitrogen balance measurements taken before *N. brasiliensis* infection (Trial 1)

Results are means of 7 observations

<sup>a,b,c,d</sup> means with different superscripts within a row are significantly different  
p<0.05.

<sup>1</sup>P.E.R. Protein Efficiency Ratio      Weight gain (g) / Protein ingested (g)  
(Protein ingested = Nitrogen eaten x 6.25)

<sup>2</sup>A.B.V. Apparent Biological Value

$$((N_{\text{eaten}} - (N_{\text{faeces}} + N_{\text{urine}})) / (N_{\text{eaten}} - N_{\text{faeces}})) \times 100$$

<sup>3</sup>A.D. Apparent Digestibility

$$(N_{\text{eaten}} - N_{\text{faeces}}) / N_{\text{eaten}} \times 100$$

| Diet                      | H                    | H + eQT              | L                    | L + eQT            | s.e.d.<br>(42 d.f.) |
|---------------------------|----------------------|----------------------|----------------------|--------------------|---------------------|
| Water (g/d)               | 36.81 <sup>a</sup>   | 33.92 <sup>a</sup>   | 47.91 <sup>a</sup>   | 26.82 <sup>b</sup> | 7.429               |
| N intake (g/d)            | 0.671 <sup>a</sup>   | 0.634 <sup>b</sup>   | 0.250 <sup>c</sup>   | 0.269 <sup>c</sup> | 0.0169              |
| Faeces (gDM/d)            | 2.19 <sup>a</sup>    | 2.68 <sup>b</sup>    | 2.13 <sup>a</sup>    | 2.66 <sup>b</sup>  | 0.144               |
| % N faeces<br>(gN/100gDM) | 1.888 <sup>a</sup>   | 4.355 <sup>b</sup>   | 1.537 <sup>c</sup>   | 3.474 <sup>d</sup> | 0.0688              |
| N faeces (g/d)            | 0.041 <sup>a</sup>   | 0.117 <sup>b</sup>   | 0.033 <sup>a</sup>   | 0.092 <sup>c</sup> | 0.0040              |
| Urine (g/d)               | 14.99 <sup>a,b</sup> | 12.52 <sup>a,b</sup> | 20.88 <sup>a</sup>   | 7.61 <sup>b</sup>  | 4.329               |
| % N urine<br>(gN/100g)    | 3.091 <sup>a</sup>   | 3.389 <sup>a</sup>   | 0.441 <sup>b</sup>   | 0.580 <sup>b</sup> | 0.4319              |
| N urine (g/d)             | 0.378 <sup>a</sup>   | 0.323 <sup>b</sup>   | 0.057 <sup>c</sup>   | 0.034 <sup>c</sup> | 0.0147              |
| Total N excreted<br>(g/d) | 0.419 <sup>a</sup>   | 0.439 <sup>a</sup>   | 0.089 <sup>c</sup>   | 0.125 <sup>d</sup> | 0.0157              |
| N retained (g/d)          | 0.251 <sup>a</sup>   | 0.194 <sup>b</sup>   | 0.161 <sup>b,c</sup> | 0.144 <sup>c</sup> | 0.0172              |
| P.E.R. <sup>1</sup>       | 1.171 <sup>a</sup>   | 0.915 <sup>a</sup>   | 2.595 <sup>b</sup>   | 1.960 <sup>c</sup> | 0.2019              |
| A.B.V. <sup>2</sup>       | 39.89 <sup>a</sup>   | 37.19 <sup>a</sup>   | 73.73 <sup>c</sup>   | 80.90 <sup>d</sup> | 2.970               |
| A.D. <sup>3</sup>         | 93.84 <sup>a</sup>   | 81.50 <sup>b</sup>   | 86.92 <sup>c</sup>   | 65.83 <sup>d</sup> | 0.740               |



### *Nitrogen balance measurements pre-infection*

Mean nitrogen balance measurements are shown in Table 3.3, taken from day -7 to day 0 (infection). Water consumption recorded throughout the balance period was highly variable within a dietary treatment. The inclusion of dietary eQT decreased water consumption, the decrease was non-significant for rats fed H+eQT but significant for L+eQT fed rats compared to the control rats ( $p<0.01$ ).

The mean daily total nitrogen intake of the 4 dietary groups indicated that diet H-fed rats consumed approximately 2.5-fold more dietary nitrogen (equivalent to the difference in dietary protein concentration). The inclusion of eQT in diet H resulted in less nitrogen being consumed than control rats ( $p<0.05$ ) due to a non-significant reduction in feed intake. The inclusion of quebracho tannin (eQT & uQT) into diet L did not produce any significant differences in feed intake ( $p>0.2$ ).

Total daily faecal output (expressed as g DM/d) was not influenced by dietary protein concentration. The inclusion of dietary eQT increased total faecal output by approximately 25%, and increased the nitrogen content of the faeces by almost 3-fold compared to control-fed rats ( $p<0.001$ ). Thus, the total faecal nitrogen output was significantly greater from the eQT-fed rats.

Urine volume tended to decrease with dietary eQT inclusion. This decline was significant for those rats fed diet L ( $p<0.01$ ) due to a reduced water intake. The nitrogen content of urine was greater from rats fed diet H compared to L-fed rats ( $p<0.001$ ). The inclusion of eQT to diet H reduced the percentage nitrogen in the urine ( $p<0.001$ ) and non-significantly in diet L-fed rats. Total nitrogen excreted in the urine over 24h was approximately 6-fold greater from H-fed rats compared to L-fed rats.

The total nitrogen excreted in both faeces and urine was significantly greater from rats fed diet H ( $p<0.001$ ) than rats fed diet L. Total nitrogen excretion was increased

by the inclusion of dietary eQT in diet L ( $p<0.05$ ) and non-significantly in diet H ( $p>0.2$ ).

Nitrogen retained in the body of the animals was calculated as the difference between total nitrogen output and nitrogen intake. Rats fed a higher dietary protein concentration retained a greater quantity of nitrogen. The inclusion of dietary eQT significantly reduced the nitrogen retention by rats fed diet H ( $p<0.01$ ), the decline was non-significant in diet L ( $p>0.2$ ).

Protein efficiency ratio was significantly improved in diet L-fed rats ( $p<0.001$ ), with a decrease seen due to eQT inclusion (diet H,  $p>0.2$ ; diet L,  $p<0.01$ ). The apparent biological value of the diets, the proportion of food protein which can be utilised by the animal for synthesising body tissues and compounds (McDonald et al., 1995) was almost 2-fold greater for diet L, the inclusion of eQT in diet L further increased the apparent biological value of the diet ( $p<0.05$ ). Finally apparent digestibility was calculated, this being significantly reduced in diet L ( $p<0.001$ ) and further depressed by dietary eQT ( $p<0.001$ ).

#### *Nitrogen balance measurements post-infection*

Data for mean nitrogen balance measurements taken between days 0 and 5 p.i. are shown in Table 3.4. Dietary effects (high and low protein, with and without eQT) were generally unaltered following nematode infection. *N. brasiliensis* infection did not significantly alter total daily faecal and urine excretion or nitrogen content of those excretions. Reductions were observed in protein efficiency ratio post-infection for rats fed diet L  $\pm$  eQT fed rats ( $p<0.01$ ) and non-significantly for those animals diet H + eQT ( $p<0.1$ ). Apparent biological value of diet L was reduced post-infection ( $p<0.01$ ), no significant differences were observed for the remaining dietary groups. Apparent digestibility was not affected by nematode infection.



**Table 3.4** Effect of high and low dietary protein concentration, with and without eQT, on mean daily nitrogen balance measurements taken after *N. brasiliensis* infection (Trial 1)

Results are means of 5 observations

<sup>a,b,c,d</sup> means with different superscripts within a row are significantly different p<0.05.

<sup>1</sup>P.E.R. Protein Efficiency Ratio       $\text{Weight gain (g)} / \text{Protein ingested (g)}$   
(Protein ingested = Nitrogen eaten x 6.25)

<sup>2</sup>A.B.V. Apparent Biological Value  
 $(\text{N eaten} - (\text{N faeces} + \text{N urine})) / (\text{N eaten} - \text{N faeces}) \times 100$

<sup>3</sup>A.D. Apparent Digestibility  
 $(\text{N eaten} - \text{N faeces}) / \text{N eaten} \times 100$

| Diet                      | H                   | H + eQT              | L                  | L + eQT            | s.e.d.<br>(42 d.f.) |
|---------------------------|---------------------|----------------------|--------------------|--------------------|---------------------|
| Water (g/d)               | 44.71               | 31.66                | 40.27              | 27.31 <sup>a</sup> | 7.429               |
| N intake (g/d)            | 0.658 <sup>a</sup>  | 0.606 <sup>b</sup>   | 0.235 <sup>c</sup> | 0.264 <sup>c</sup> | 0.0169              |
| Faeces (gDM/d)            | 2.30 <sup>a,b</sup> | 2.55 <sup>b,c</sup>  | 2.09 <sup>a</sup>  | 2.62 <sup>c</sup>  | 0.144               |
| % N faeces<br>(gN/100g)   | 1.923 <sup>a</sup>  | 4.347 <sup>b</sup>   | 1.643 <sup>c</sup> | 3.498 <sup>d</sup> | 0.0688              |
| N faeces (g/d)            | 0.043 <sup>a</sup>  | 0.111 <sup>b</sup>   | 0.034 <sup>c</sup> | 0.091 <sup>d</sup> | 0.0040              |
| Urine (g/d)               | 18.73 <sup>a</sup>  | 10.48 <sup>a,b</sup> | 15.71 <sup>a</sup> | 6.30 <sup>b</sup>  | 4.329               |
| % N urine<br>(gN/100g)    | 2.608 <sup>a</sup>  | 3.559 <sup>b</sup>   | 0.559 <sup>c</sup> | 0.885 <sup>c</sup> | 0.4319              |
| N urine (g/d)             | 0.396 <sup>a</sup>  | 0.323 <sup>b</sup>   | 0.070 <sup>c</sup> | 0.039 <sup>c</sup> | 0.0147              |
| Total N excreted<br>(g/d) | 0.438 <sup>a</sup>  | 0.434 <sup>a</sup>   | 0.104 <sup>b</sup> | 0.130 <sup>b</sup> | 0.0157              |
| N retained (g/d)          | 0.216 <sup>a</sup>  | 0.173 <sup>b</sup>   | 0.131 <sup>c</sup> | 0.133 <sup>c</sup> | 0.0172              |
| P.E.R. <sup>1</sup>       | 0.838 <sup>a</sup>  | 0.806 <sup>a</sup>   | 1.916 <sup>b</sup> | 1.414 <sup>c</sup> | 0.2019              |
| A.B.V. <sup>2</sup>       | 35.17 <sup>a</sup>  | 34.25 <sup>a</sup>   | 64.87 <sup>b</sup> | 76.84 <sup>c</sup> | 2.970               |
| A.D. <sup>3</sup>         | 93.45 <sup>a</sup>  | 81.65 <sup>b</sup>   | 85.50 <sup>c</sup> | 65.51 <sup>d</sup> | 0.740               |



### 3.3.1.3 Worm burden and eggs in utero

The effect of dietary protein concentration and eQT inclusion on the burden of adult *N. brasiliensis* worms recovered from the small intestine of rats fed different diets is shown in Figure 3.2. The increase in dietary protein concentration from 10% (w/w) casein to 25% (w/w) casein did not alter the worm burden recovered. The inclusion of dietary eQT reduced nematode burdens in both dietary protein concentrations (H & L) by approximately 50 % ( $p<0.001$ ).

The number of eggs *in utero* counted from 5 randomly chosen female worms from each rat were unaffected by dietary protein concentration. The inclusion of dietary eQT reduced the eggs *in utero* of the female worms examined by approximately 25% at both dietary protein concentrations ( $p<0.001$ ).

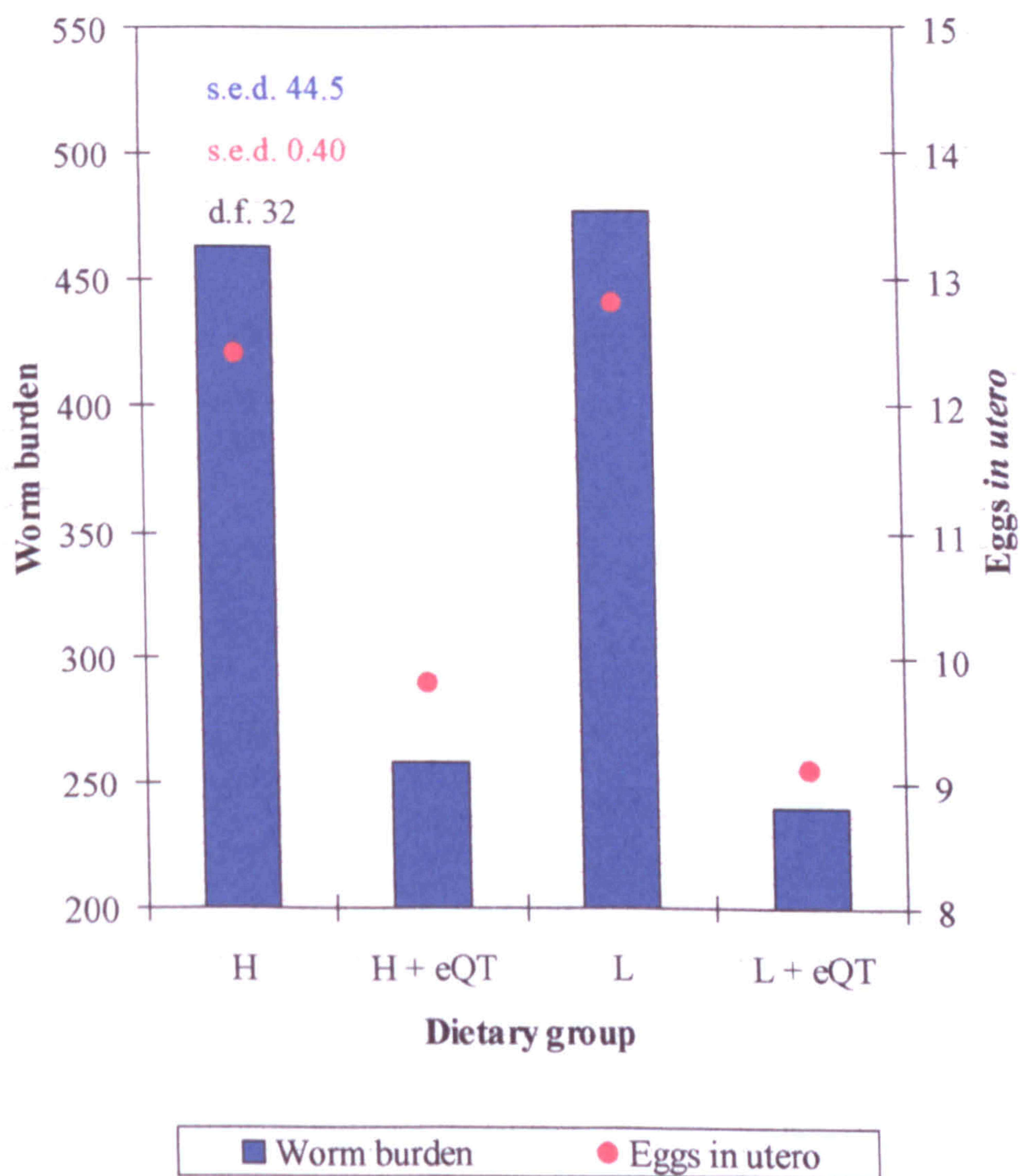
### 3.3.2 Trial 2 investigating the effect of high and low dietary protein concentration, with and without quebracho tannin, on the establishment of *N. brasiliensis* in the small intestine of rats

#### 3.3.2.1 Liveweight and feed intake

The mean bodyweights of the animals fed the six dietary treatments are shown in Figure 3.3. Growth was greatest by rats fed diet H with and without tannin inclusion (eQT and uQT) compared to the three groups fed the corresponding diets L with and without tannin inclusion. Dietary quebracho tannin tended to reduce daily growth rates, however this was not consistent for either eQT or uQT (Table 3.5). Dietary treatments (protein concentration, tannin inclusion and tannin type) did not significantly alter mean feed intakes.

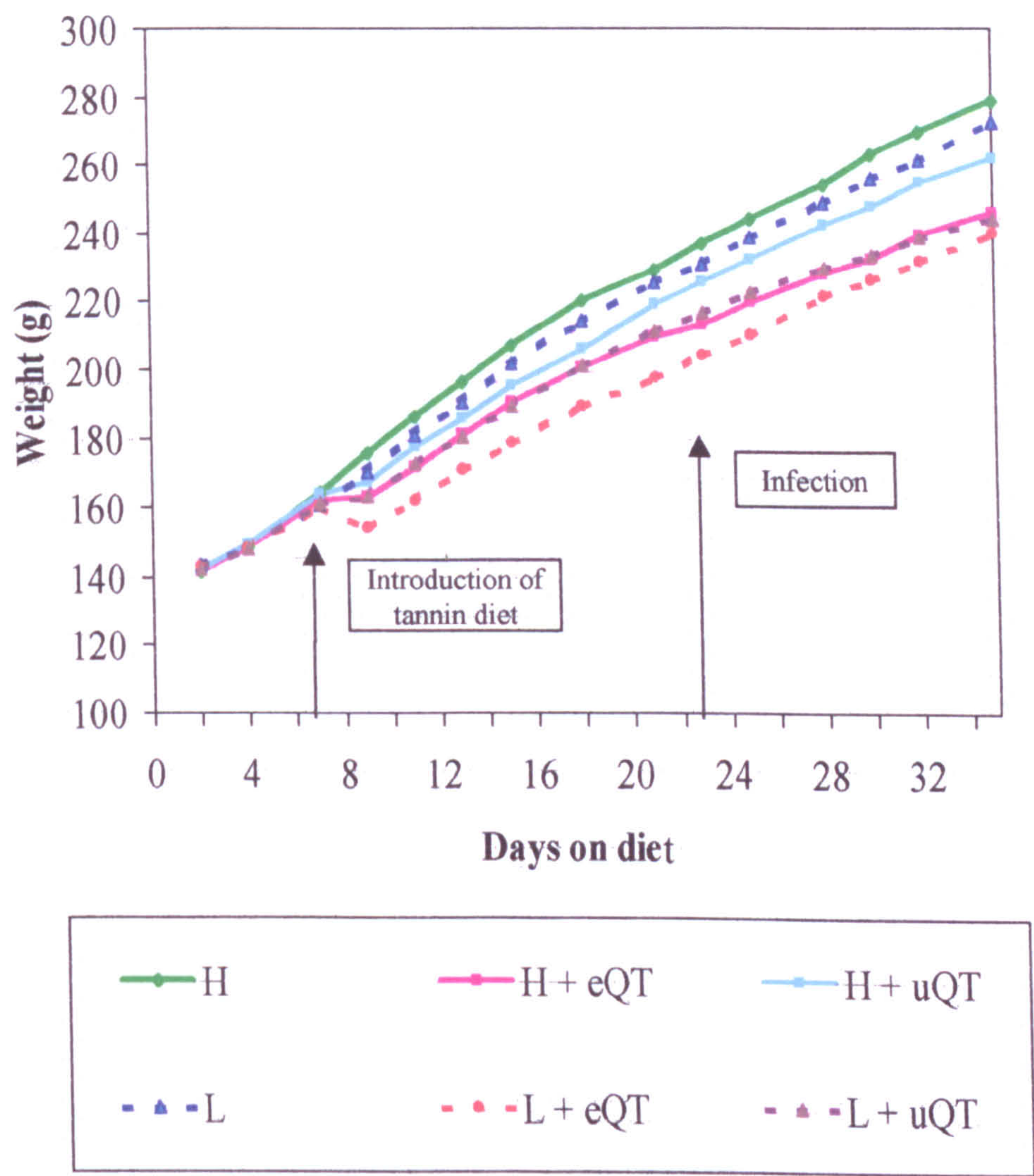


**Figure 3.2** Effect of high and low dietary protein concentration, with and without eQT, on the number of adult *N. brasiliensis* worms recovered from the small intestine 5 days p.i. and the number of eggs observed *in utero* from randomly chosen female worms (Trial 1)





**Figure 3.3** Effect of high and low dietary protein concentration, with and without dietary quebracho tannin, on growth of *N. brasiliensis* infected rats (Trial 2)





**Table 3.5** Effect of high and low dietary protein concentration, with and without quebracho tannin, on growth rate and daily feed intake of rats (Trial 2)

|                         | Growth rate (g/d)   | Feed Intake (g/d) |
|-------------------------|---------------------|-------------------|
| <b>H</b>                | 3.72 <sup>a</sup>   | 20.5              |
| <b>H + eQT</b>          | 2.88 <sup>b</sup>   | 19.9              |
| <b>H + uQT</b>          | 3.55 <sup>a</sup>   | 19.4              |
| <b>L</b>                | 3.71 <sup>a</sup>   | 20.7              |
| <b>L + eQT</b>          | 3.30 <sup>a,b</sup> | 20.4              |
| <b>L + uQT</b>          | 2.93 <sup>b</sup>   | 20.6              |
| <b>s.e.d. (24 d.f.)</b> | 0.266               | 1.44              |

<sup>a,b,c,d</sup>means with different superscripts within a column are significantly different p<0.05.

Results are means of 8 observations from the introduction of dietary tannin into appropriate animal groups to 5 days p.i.



#### **3.3.2.2 Worm burden and eggs in utero**

The worm burdens recovered from the rats 5 days p.i. are shown in Figure 3.4. Control rats fed diets H and L had the largest worm burden recovered from the small intestine. The inclusion of tannin (eQT & uQT) at both dietary protein concentrations reduced worm burdens ( $p < 0.05$ ). Reductions in worm burden at day 5 p.i. were similar from eQT and uQT-fed rats. Twenty female worms from each rat were randomly chosen and the numbers of egg *in utero* counted and recorded. In contrast to the results reported in trial 1 the numbers of eggs *in utero* did not appear to be effected by dietary treatment ( $p > 0.2$ ).

### **3.3.3 Trial 3 investigating the effect of high and low dietary protein concentration, with and without quebracho tannin on the daily faecal egg output from *N. brasiliensis* infected rats**

#### **3.3.3.1 Liveweight and feed intake**

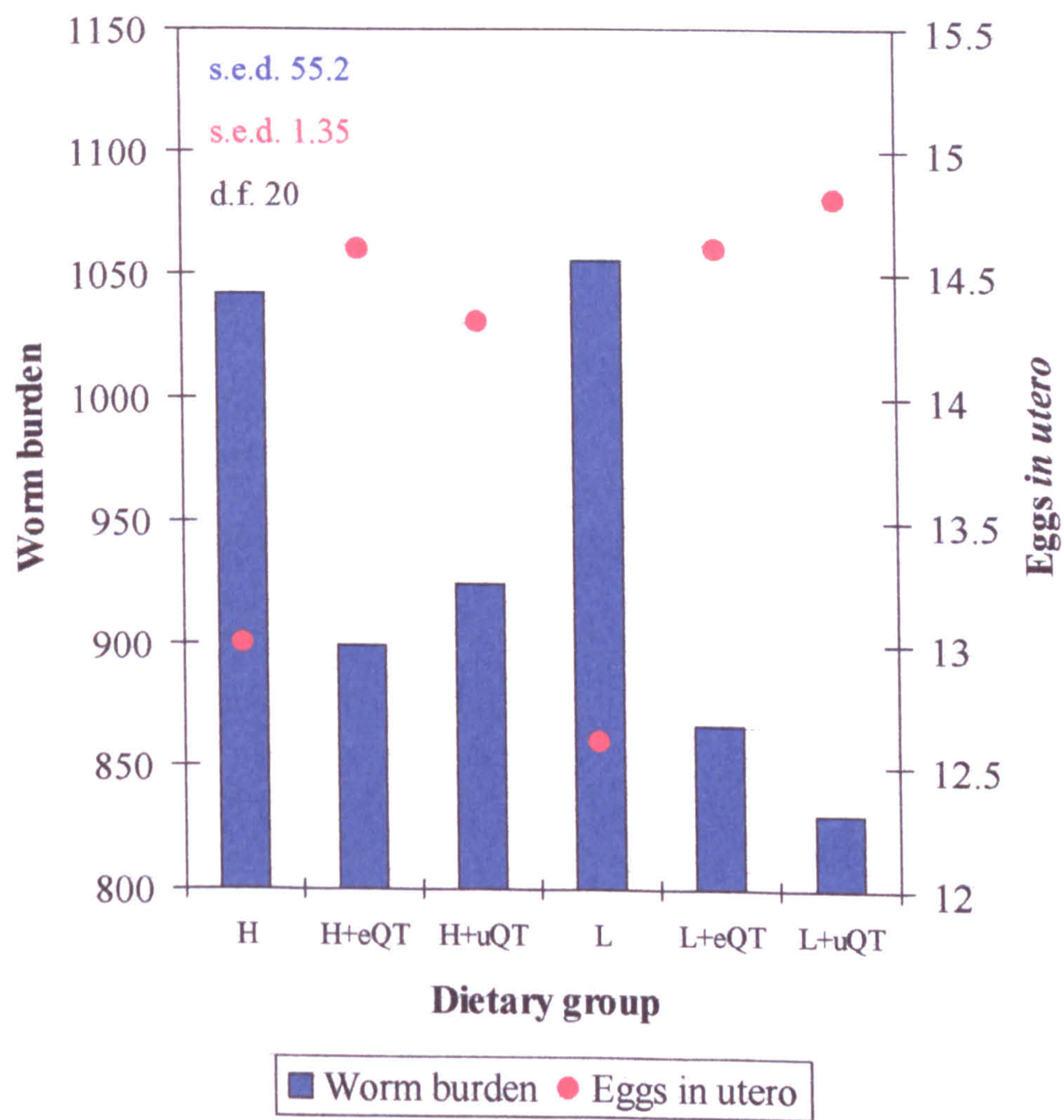
The mean bodyweights of the rats fed the six dietary treatments are shown in Figure 3.5. Growth was greatest in rats fed diet H with and without tannin inclusion (eQT and uQT) compared to the three groups fed the corresponding diet L with and without tannin inclusion. No significant reductions in growth rate due to the inclusion of dietary tannin (eQT and uQT, see Table 3.6) were seen.

Mean feed intakes of the six dietary treatment groups, taken from the introduction of tannin diets until 5 days p.i., are shown in Table 3.6. The inclusion of tannin (eQT & uQT) appeared to have no consistent influence on feed intake at either dietary protein concentration.

Bodyweight and feed intakes were similar following the inclusion of uQT or eQT.

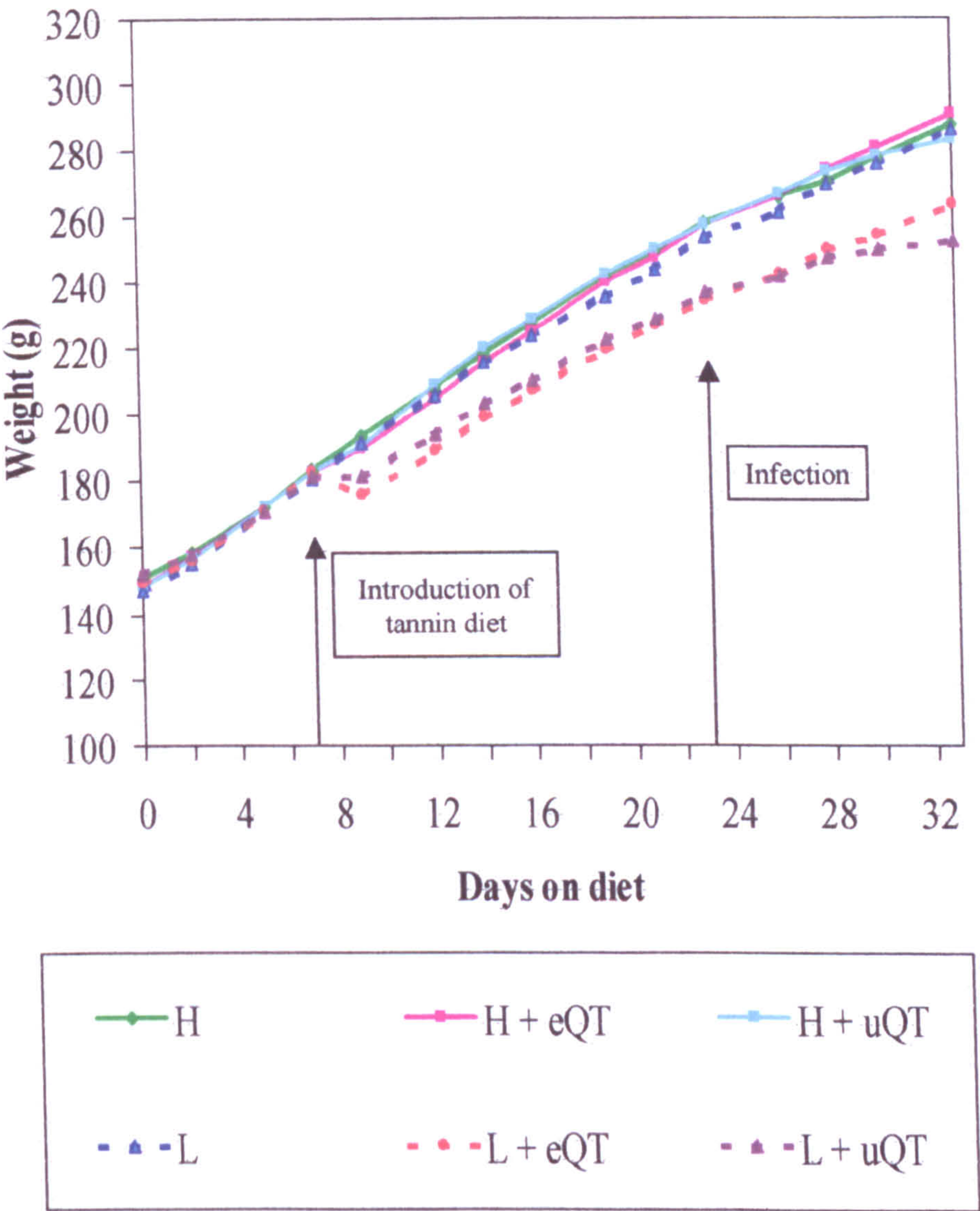


**Figure 3.4** Effect of high and low dietary protein concentration, with and without dietary quebracho tannin, on the number of adult *N. brasiliensis* worms recovered from the small intestine on day 5 p.i. and the number of eggs observed *in utero* from randomly chosen female worms (Trial 2)





**Figure 3.5** Effect high and low dietary protein concentration, with and without dietary quebracho tannin, on growth of *N. brasiliensis* infected rats (Trial 3)





**Table 3.6** Effect of high and low dietary protein concentration, with and without quebracho tannin, on growth rate and daily feed intake of rats (Trial 3)

|                  | Growth rate (g/d)   | Feed Intake (g/d)   |
|------------------|---------------------|---------------------|
| H                | 3.87 <sup>a,b</sup> | 20.0 <sup>a,b</sup> |
| H + eQT          | 4.24 <sup>a</sup>   | 20.4 <sup>a,b</sup> |
| H + uQT          | 3.98 <sup>a,b</sup> | 19.5 <sup>b</sup>   |
| L                | 4.02 <sup>a,b</sup> | 21.9 <sup>a</sup>   |
| L + eQT          | 3.47 <sup>b</sup>   | 21.3 <sup>a,b</sup> |
| L + uQT          | 3.05 <sup>b,c</sup> | 20.8 <sup>a,b</sup> |
| L-L + eQT        | 3.03 <sup>b,c</sup> | 20.4 <sup>a,b</sup> |
| s.e.d. (35 d.f.) | 0.317               | 1.06                |

<sup>a,b,c,d</sup> means with different superscripts within a column are significantly different p<0.05.

Results are means of 12 observations from the introduction of dietary tannin into appropriate animal groups to 10 days p.i.



### *3.3.3.2 Faecal egg counts and total faecal collection*

Table 3.7 shows mean quantities of fresh and dry faecal matter passed per day, eggs per gram of dry faeces and total eggs passed each day. All values are means of 6 observations taken from day 5 p.i. through to day 10 p.i.

The mean mass of fresh faeces passed by rats varied with dietary treatment. Irrespective of dietary protein concentration, tannin-fed rats (eQT and uQT) passed a greater quantity of faeces than control-fed rats. Diet H + eQT fed rats passed the greatest quantity of faeces, approximately 75% more than the diet H-fed rats ( $p < 0.001$ ). Inclusion of uQT in diet H increased faecal mass by 48% ( $p < 0.01$ ). Similarly, inclusion of dietary tannin increased faecal output in L-fed rats, dietary eQT increased faecal output by approximately 30% ( $p < 0.05$ ), while dietary uQT increased faecal output by 20% ( $p < 0.2$ ). Changing the diet from L to L + eQT at nematode infection increased faecal output to a similar mass as rats fed L + eQT prior to nematode infection. The increase in faecal output due to dietary tannin was greater from eQT-fed rats compared to rats fed uQT.

The dry matter content of faecal pellets was significantly higher in control-fed rats (H & L) compared to tannin-fed rats (eQT & uQT;  $p < 0.05$ ). In both diet L and H-fed rats the inclusion of eQT reduced the dry matter of the faeces to a greater extent than dietary uQT ( $p < 0.01$ ).

When collecting the faeces it was noted that in addition to tannin-fed rats passing faecal pellets which were stained with tannin (red-brown in colour compared to the greyish faeces from control-fed rats) there was also a mucus 'membrane' surrounding the fresh faecal pellet. This observation of additional mucus secretion tended to be more apparent in faecal pellets passed by the eQT-fed rats compared to rat fed uQT. The reduced faecal dry matter of tannin-fed rats may have been partly due to the increased mucus content, in addition these faeces tended to take longer to

**Table 3.7** Effect of high and low dietary protein concentration, with and without quebracho tannin, on total daily faecal output and the number of nematode eggs passed in the faeces (Trial 3)

|                  | Faeces<br>(g/d)     | Faeces<br>(g DM/d)    | DM<br>(%)           | EPG<br>(DM)       | Total Eggs/d      |
|------------------|---------------------|-----------------------|---------------------|-------------------|-------------------|
| H                | 3.04 <sup>a</sup>   | 2.51 <sup>a,c</sup>   | 80.3 <sup>a</sup>   | 1147 <sup>a</sup> | 2474 <sup>a</sup> |
| H + eQT          | 5.29 <sup>b</sup>   | 3.25 <sup>b</sup>     | 59.5 <sup>d</sup>   | 801 <sup>a</sup>  | 2801 <sup>a</sup> |
| H + uQT          | 4.49 <sup>c</sup>   | 3.12 <sup>a,b</sup>   | 66.9 <sup>c</sup>   | 631 <sup>a</sup>  | 1769 <sup>a</sup> |
| L                | 3.17 <sup>a</sup>   | 2.76 <sup>a,b,c</sup> | 84.5 <sup>a</sup>   | 2516 <sup>b</sup> | 5177 <sup>b</sup> |
| L + eQT          | 4.16 <sup>c</sup>   | 2.71 <sup>a,b,c</sup> | 63.3 <sup>c,d</sup> | 663 <sup>a</sup>  | 1697 <sup>a</sup> |
| L + uQT          | 3.79 <sup>a,c</sup> | 2.86 <sup>a,b,c</sup> | 74.0 <sup>b</sup>   | 1041 <sup>a</sup> | 2481 <sup>a</sup> |
| L-L + eQT        | 4.26 <sup>c</sup>   | 2.43 <sup>c</sup>     | 58.7 <sup>d</sup>   | 628 <sup>a</sup>  | 1700 <sup>a</sup> |
| s.e.d. (35 d.f.) | 0.451               | 0.330                 | 2.53                | 458.3             | 1018.9            |

<sup>a,b,c,d</sup> means with different superscripts within a column are significantly different p<0.05.

Results are means of 6 observations from 5 days p.i to day 10 p.i.



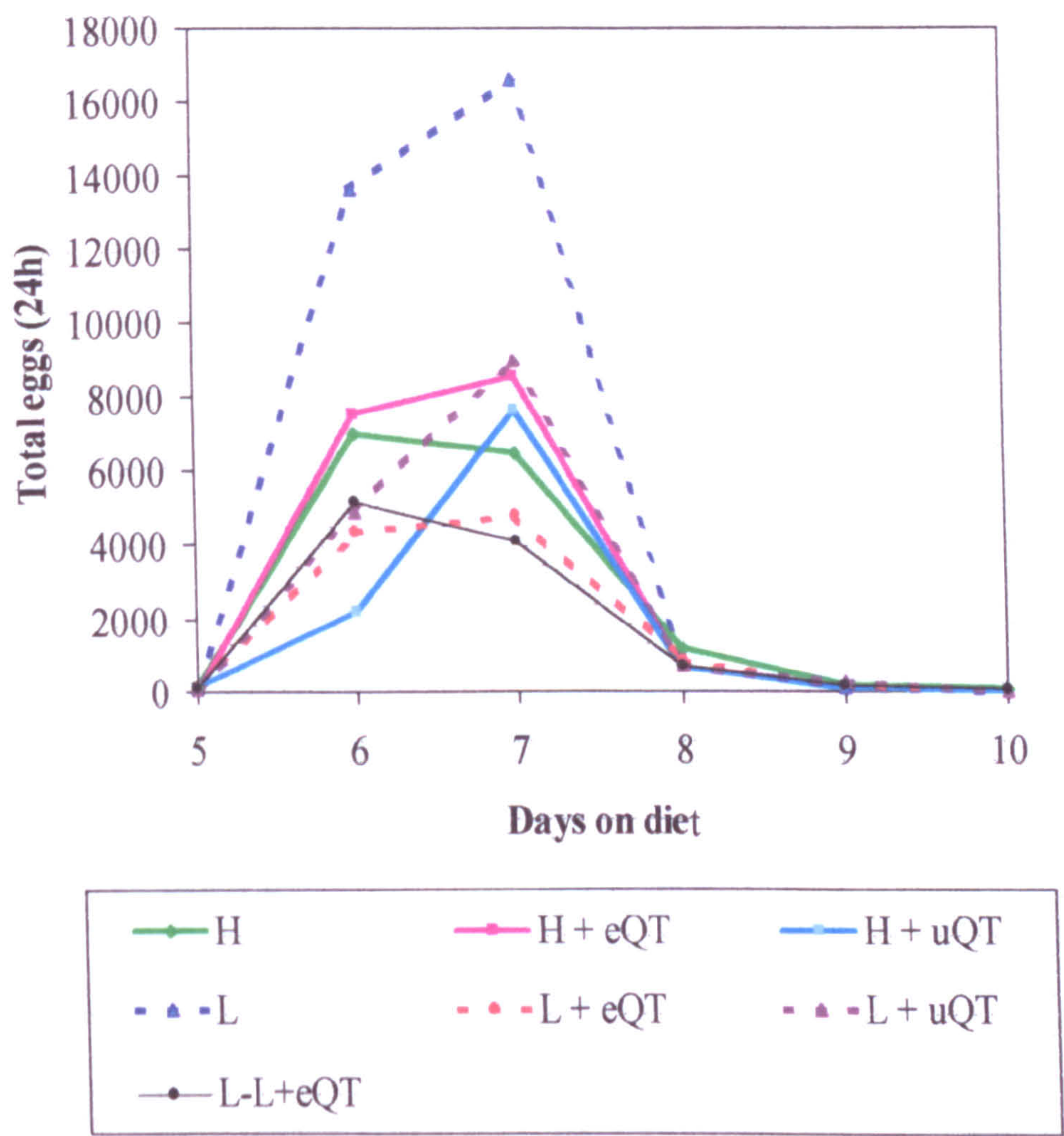
soak in water for egg counting. Consequently total daily dry faecal matter excreted by diet L-fed rats was not significantly different between control and tannin-fed rats. The dry faeces passed during a 24h period by diet H + eQT rats was still significantly greater than diet H-fed rats ( $p < 0.05$ ).

The pattern of egg production is shown in Figure 3.6. *N. brasiliensis* eggs were first seen in rat faeces 5 days p.i., numbers peaked on day 7 p.i. and fell to negligible levels by day 9 p.i. Table 3.7 contains mean total faecal egg output for each dietary treatment. The numbers of eggs passed in one gram of dry faeces by rats fed diet L were significantly greater ( $p < 0.01$ ) than those in faeces passed by those rats fed dietary quebracho tannin (L + eQT, L + uQT & L-L + eQT). The number of eggs passed in the faeces over a 24h period was calculated by multiplying the EPG figure by total dry faecal output. The difference in faecal output between dietary groups slightly altered total egg output. The only significant difference between dietary treatments was again that rats fed diet L passed the largest number of eggs indicating the heaviest infection level. There was a significant decrease in egg output from the inclusion of quebracho tannin in diet L (L + eQT, L + uQT & L-L + eQT;  $p < 0.05$ ). Results from rats fed diet L where eQT was included into the diet before and at infection were very similar indicating the inclusion of eQT into the diet is not required prior to infection to have a negative effect on parasite numbers. The decline in egg numbers was not as marked in rats fed diet L + uQT compared to those fed diet L + eQT ( $p > 0.1$ ). There were no significant differences between egg outputs from diet H-fed rats with or without dietary tannin.

Total egg output followed a cubic pattern when analysed for day x diet interaction (see Table 3.8). The egg output on day 5 p.i., when eggs were first observed in the faeces, was low in all dietary groups ( $p > 0.1$ ). By day 6 p.i. diet L-fed rats were excreting significantly more eggs than the other dietary groups ( $p < 0.01$ ). Rats fed diet H + uQT passed fewer eggs than any other group ( $p < 0.05$ ). No significant differences were seen between rats fed dietary tannins in diet L. On day 7 p.i. L-fed rats were still excreting the largest number of eggs ( $p < 0.001$ ). Numbers of eggs passed by rats fed diet H + uQT were no longer statistically different from other diet



**Figure 3.6** Effect of high and low dietary protein concentration, with and without dietary quebracho tannin, on the total daily fresh faecal egg output from *N. brasiliensis* infected rats (Trial 3)



Total faecal egg counts calculated from EPG figures x total fresh faeces excreted in that 24h period



**Table 3. 8** Effect of high and low dietary protein concentration, with and without quebracho tannin, on the total number of nematode eggs passed over a 24h period (Trial 3)

| Days p.i. | 5   | 6                  | 7                  | 8    | 9   | 10 |
|-----------|-----|--------------------|--------------------|------|-----|----|
| H         | 108 | 6965 <sup>b</sup>  | 6417 <sup>b</sup>  | 1156 | 155 | 45 |
| H + eQT   | 7   | 7520 <sup>b</sup>  | 8502 <sup>b</sup>  | 646  | 128 | 4  |
| H + uQT   | 114 | 2176 <sup>c</sup>  | 7615 <sup>b</sup>  | 612  | 75  | 12 |
| L         | 101 | 13587 <sup>a</sup> | 16606 <sup>a</sup> | 680  | 87  | 2  |
| L + eQT   | 46  | 4339 <sup>b</sup>  | 4755 <sup>b</sup>  | 851  | 150 | 52 |
| L + uQT   | 69  | 4900 <sup>b</sup>  | 8960 <sup>b</sup>  | 686  | 266 | 3  |
| L-L + eQT | 83  | 5152 <sup>b</sup>  | 4054 <sup>c</sup>  | 707  | 160 | 42 |

<sup>a,b</sup>means with different values within a column are significantly different p<0.05.

The standard error of difference for day x diet interaction was 2302.6 with 210 degrees of freedom.

H-fed rats, the inclusion of eQT in diet L (before and at infection) tended to reduced the number of eggs excreted compared to rats fed diet L + uQT ( $p < 0.1$ ). The eggs recovered in the faeces on day 8 p.i. had all declined to low levels and no significant differences were observed between treatments ( $p > 0.1$ ).



### 3.4 PRELIMINARY DISCUSSION

The aims of this chapter were to investigate the effect of the inclusion of quebracho tannin in the diet of rats, and its interaction with high and low dietary protein concentrations on the establishment and persistence of *N. brasiliensis* in the small intestine.

Production parameters were measured through growth rates, feed intakes and nitrogen balance. Dietary quebracho tannin had an adverse effect on growth rates of rats at both high and low dietary protein concentrations. Nitrogen excretion was increased from tannin-fed animals and nitrogen retention decreased. Feed intake was not significantly affected by dietary tannin inclusion.

*N. brasiliensis* infection in rats usually leads to depressed nutrient absorption by the small intestine, especially in those regions infected by the parasites (Symons, 1969; Scofield, 1977, 1980). Cheema & Scofield (1982) observed significant changes in rat small intestine and colon mucosa 10 days after *N. brasiliensis* infection. Flattening, irregularities in shape and fusion of villi and shorter and more irregular distribution of microvilli were seen along with increased goblet cell activity (main secretors of mucin in the gastrointestinal tract, Specian & Oliver, 1991). Structural damage in the duodenum and proximal ileum was seen after infection of 40 larvae. The distal ileum and colon showed significant changes after infections of 200 or more larvae. Nawa (1979) observed increased mucosal permeability when *N. brasiliensis* was present in the intestine of Wistar rats, the degree of permeability being proportional to the worm burden. Reduced nutrient absorption, increased goblet cell activity, hence increased mucus secretion, and increased mucosal permeability would be anticipated to result in increased nitrogen excretion by infected rats in rat trial 1. However, nematode infection had little effect on the parameters measured during the nitrogen balance study. A possible explanation for this may be that nitrogen balance measurement ceased on day 5 p.i. The worms had only been present in the small intestine from approximately day 2 p.i. and only just attained sexual maturity when

recovered from the small intestine on day 5 p.i. Their presence in the small intestine during days 5, 6 and 7 p.i. may have resulted in greater damage and increased nitrogen excretion. Protein efficiency ratio and apparent biological value of the feed were depressed after infection indicating that *N. brasiliensis* infection altered digestion, absorption or utilisation of the diet. The absence of consistent reductions in nitrogen retention after infection suggests that nitrogen digestion and absorption were not affected by the presence of parasites, therefore the influence of infection maybe on the utilisation of dietary nitrogen possibly due to the initiation of an immune response against the parasite. Frandsen (1985) observed significant increases in faecal nitrogen in rats infected with 2000 L<sub>3</sub> *N. brasiliensis* infected rats but this was only apparent from day 10 p.i. onwards. The absence of any difference in faecal nitrogen in the present study is likely to be a consequence of the short time period over which nitrogen balance was studied and also the smaller L<sub>3</sub> dose injected (1000 L<sub>3</sub> *N. brasiliensis*).

The inclusion of dietary quebracho tannin reduced *N. brasiliensis* infection. A significant reduction in the number of worms establishing in the gut was observed in tannin-fed rats and was unaffected by dietary protein concentration. This suggests that increasing the protein supply to the host does not affect the establishment of infective larvae as observed by others (Lunn *et al.*, 1988). When the female worms from the tannin-fed rats were examined fewer eggs were observed *in utero* suggesting that dietary tannin delayed sexual maturity. The persistence of infection was monitored by total daily faecal egg counts. Rats fed the high protein diet excreted fewer nematode eggs in the faeces than low protein-fed rats. The inclusion of dietary quebracho also reduced egg counts compared to low protein-fed controls to concentrations comparable with high protein-fed controls. Dietary quebracho tannin inclusion in the high protein diet did not further reduce faecal egg counts below those observed from low protein tannin-fed rats and high protein-fed controls.

In summary, the inclusion of quebracho tannin in rat diets reduced *N. brasiliensis* establishment and persistence in the small intestine compared to low protein-fed



controls. Both extracted and untreated quebracho tannin (eQT & uQT) were effective at reducing worm burden at day 5 p.i. and faecal egg counts. This suggests that the active component in eQT is not a residue from the chemical extraction process as it is also present in the untreated quebracho tannin.

The results from this chapter form the basis for the first sheep study. As the active component eQT appears to be present in uQT, eQT will continue to be used as the model condensed tannin. The effect of dietary quebracho tannin on small intestinal parasite infection in the sheep maintained on energy restricted diets is reported in chapter 4.

**CHAPTER 4**  
**EFFECT OF DIETARY QUEBRACHO TANNIN ON**  
**NEMATODE INFECTED LAMBS MAINTAINED ON A FEED**  
**INTAKE-RESTRICTED DIET**  
**(SHEEP STUDY 1)**

**4.1 INTRODUCTION**

The climate of the tropics and subtropics tends to favour the development and survival of animal parasites, increasing the likelihood of parasitic infection of animals in these areas compared to temperate regions (Waller, 1997). Anthelmintic treatment to control parasitic disease is often not feasible (Waller, 1997). The drugs may be unaffordable, of such inferior quality that they are not used and their continued over-use and mis-use is leading to a state of total chemotherapeutic failure (Waller, 1997). Animals in tropical and subtropical environments often feed extensively on browse species containing condensed tannins (Tolera *et al.*, 1997). Inclusion of these compounds in monogastric diets is considered to be anti-nutritional due to their capacity to bind to proteins and carbohydrates (Jansman, 1993). Conversely, low concentrations in ruminant diets can be beneficial, preventing bloat and increasing the supply of bypass protein to the small intestine through binding to dietary protein and preventing ruminal degradation (Barry *et al.*, 1986b). It has long been recognised that improved levels of nutrition promote the ability of the animal to cope with internal parasites (Gibson, 1963, Dobson & Bawden, 1974; Lunn *et al.*, 1988; Poppi *et al.*, 1990) while undernutrition depresses the immunoreactivity of the host (Wan *et al.*, 1989). Increasing the supply of intestinal protein to the infected animal can improve productivity to levels similar to those of uninfected controls (Abbott *et al.*, 1988; Bown *et al.*, 1991b). Maintaining animals on high planes of nutrition in the developing world is often not possible and many suffer from malnutrition and undernutrition (Waller, 1999). The inclusion of condensed tannins in the diet may be able to protect dietary protein from rumen degradation. The resulting elevation of protein supply to the small intestine may



reduce production losses associated with internal parasitism. Evidence of improved animal performance and reduced parasite burdens has been shown by Niezen *et al.* (1995, 1998a, b) and Robertson *et al.* (1995). Trials reported in chapter 3 using the *Nippostrongylus brasiliensis*-rat model have shown that the inclusion of dietary quebracho tannin in both high and low protein diets reduced the intensity of nematode infection, irrespective of dietary protein concentration. The reduction seen in *N. brasiliensis* infection of the rat indicates that quebracho tannin must act through a mechanism other than improved protein availability at the small intestine. To investigate if these findings are applicable in the ruminant host the following study described in this chapter was conducted.

In order to try to replicate possible feeding constraints which may often be placed upon animals in the tropics and subtropics, lambs were restricted to a growth rate of 100 g/d. Pelleted dried grassmeal was chosen as the control diet, the tannin diet contained 50 g extracted quebracho tannin (eQT)/kg dried grassmeal added prior to pelleting. Once acclimatised to the diet the lambs were exposed to a trickle infection of *Trichostrongylus colubriformis* larvae to mimic field conditions. Worm infection was monitored through faecal egg counts and terminal worm burdens. Haematological parameters measured included amino acid concentrations at two separate time points and eosinophil concentrations.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Animals and Pre-trial Period

Twenty lambs, 10 castrate males and 10 females (Texel X mule), were raised in-house and maintained helminth-free from birth. The animals were individually housed from weaning (6 weeks of age) in raised slotted-floor pens. They were exposed to continuous light with free access to water and mineral licks and an ambient temperature of  $15 \pm 2^{\circ}\text{C}$  was maintained throughout.

To ensure the animals remained helminth-free, all lambs and ewes were regularly drenched with the broad-spectrum anthelmintic Parafend [Oxfendazole 2.265 %, Norbrook Laboratories, Worcestershire, UK]. Ewes were drenched just after parturition and 3 weeks later. Lambs were drenched at weaning, 12 and 18 weeks of age. Once moved to slotted floor pens any faecal material which did not pass through the flooring to the concrete below was removed every 2-3 weeks to prevent any possible infection from faecal material.

The lambs were weaned at 6 weeks of age, and from this time until the experimental diets were introduced the lambs were maintained on a standard complete pelleted diet (spec lamb; see appendix) to gain 100 g/d. Animals were weighed weekly using a weigh crate connected to a digital balance [Tru-test AG500, Tru-Test Distributors Ltd., Auckland, New Zealand]. The weigh crate was routinely calibrated before use with a standard weight of 20 kg.



#### 4.2.2 Experimental Diets

At the age of approximately 22 weeks the animals (liveweight  $32 \pm 4$  kg) were randomly divided into two groups of 10 animals (5 males and 5 females) and fed a pelleted diet of dried grassmeal alone (controls) or containing 50 g eQT/kg (tannin). The diets were fed once daily at 09.00 h on a bodyweight basis to gain 100 g/d, calculated from Agricultural and Food Research Council figures (1993) using separate equations for females and castrate males (equations 4.1 & 4.2). The metabolisable energy content of dried grass was assumed to be 10.6 MJ/kg feed (Agricultural and Food Research Council figures, 1993). Dry matter content of the diet was established as 0.95 after drying to constant weight using a 100°C oven (section 2.6.1). Dietary eQT was considered to be nutritionally inert, hence tannin-fed lambs were given an additional 5% (w/w) feed to compensate for the presence of eQT in the diet to ensure constant nutrient presentation to all animals. The quantity of diet given to each animal was re-calculated each time the animals were weighed. Any feed refusals were collected prior to the morning feed and weighed back to establish daily feed consumption. The experimental diets were introduced 4 weeks prior to the commencement of the infection period.

$$y = (0.233 \times \text{bodyweight (kg)} + 1.57) / \text{DM feed (g/g)}$$

$$\text{where kg feed given} = y / \text{ME feed (KJ/kg)}$$

**Equation 4.1** Feed allowance to restrict growth of female lambs to 100 g/d.

$$y = (0.196 \times \text{bodyweight (kg)} + 2.24) / \text{DM feed (g/g)}$$

$$\text{where kg feed given} = y / \text{ME feed (KJ/kg)}$$

**Equation 4.2** Feed allowance to restrict growth of castrate male lambs to 100 g/d.

### 4.2.3 Nematode infection

After the experimental diets had been fed for 4 weeks, animals were then exposed to a trickle infection of infective *T. colubriformis* larvae to mimic burdens gained in the field. Lambs were infected orally on 5 days/week with doses of 6000, 3000, 3000, 3000 and 6000 infective stage larvae (L<sub>3</sub>) given in 10 ml distilled water using a 10 ml syringe.

### 4.2.4 Faecal analysis

#### 4.2.4.1 Faecal egg counts

Faecal samples (approximately 10g) were regularly collected directly from the rectum once nematode eggs were observed to be present in the faeces. Collections were taken following feeding three times weekly from day 15 post infection (p.i.) up to and including a terminal egg count collected at slaughter. The number of nematode eggs present in the faeces was established following the method described in section 2.2.2.

#### 4.2.4.2 Total faecal output

Total faecal output was collected from all animals over a four-day period. During week 8 p.i. all the male lambs were housed in individual metabolism crates for a seven-day period. The first three days were used as an acclimatisation period, over the final four days total faecal output was collected and recorded every 12 h, after which the animals were returned to their pens. During week 9 p.i. the female lambs were housed in the metabolism crates. Again the first 3 days were used as an acclimatisation period with total faecal collection taken during the final four days. Faecal mass was determined for each 24 h period and a mean daily faecal output calculated for the four-day sample period.



## **4.2.4 Blood sampling**

### *4.2.4.1 Weekly blood samples*

Blood samples were taken prior to nematode infection and at weekly intervals thereafter as described in section 2.3.1. The numbers of circulating eosinophils were determined in these samples (section 2.3.2).

### *4.2.4.2 Jugular cannulation*

At two time points during the trial the animals were cannulated via the jugular vein to allow 8 blood samples to be taken at 6 hourly intervals (over a period of 48h) for the determination of plasma amino acid concentrations. The first sampling period, providing a base-line amino acid concentration, was taken just prior to the commencement of the trickle infection, once the lambs were established on the experimental diets. The second sampling period was undertaken at 44 days p.i. during what was considered to be the period of worm expulsion. In both instances plasma was collected (section 2.3.1) and stored at -40<sup>0</sup>C until analysed for amino acid concentration (section 2.3.3). Diurnal and feeding influences were removed by meaning the eight individual amino acid concentrations for that sampling period.

## **4.2.5 Slaughter protocol**

Ten weeks p.i. all animals were humanely slaughtered by conventional slaughterhouse techniques (stunned and exsanguinated). The entire intestinal tract was removed following ligation at the abomasal valve and ileal-caecal junction and the worm burden recovered as described in section 2.2.3.

#### 4.2.6 Statistical analysis

Data were analysed as a completely randomised experiment using one-way analysis of variance with diet and sex as factors [Genstat 5, Release 4.1; Lawes Agricultural Trust, Rothamsted]. Repeated measures were used to analyse changes with time within-treatments. The Greenhouse-Geisser epsilon factor obtained from repeated measures analysis was used to adjust the degrees of freedom for time x diet x sex interactions (Winer *et al.*, 1991). Data were further partitioned into linear, quadratic and cubic trends. Daily liveweight gains were obtained by linear regression and analysed using one-way analysis of variance

T-tests using the pooled standard error of difference between means were used to compare the effect of dietary eQT, sex and infection. Data were blocked for sheep. Differences were assumed to be significantly different at  $p \leq 0.05$ . Trends were considered between  $0.10 > p > 0.05$ .



## 4.3 RESULTS

### 4.3.1 Liveweight and feed intake

The growth rate of the lambs after the introduction of the experimental diets until slaughter is shown in figure 4.1. Mean bodyweights during the trial period did not alter significantly, irrespective of sex ( $p=0.123$ ) or diet ( $p=0.697$ ) and the predicted bodyweight gain of 100 g/d was achieved (Figure 4.1). No inappetence was observed during the trial with all feed being consumed within the first hour after feeding. The only animal to display inappetence was a female lamb fed the tannin diet; feed was refused 18 days after infection began and the animal was removed from the trial 20 days later. Data gained from this animal were removed from all statistical analyses.

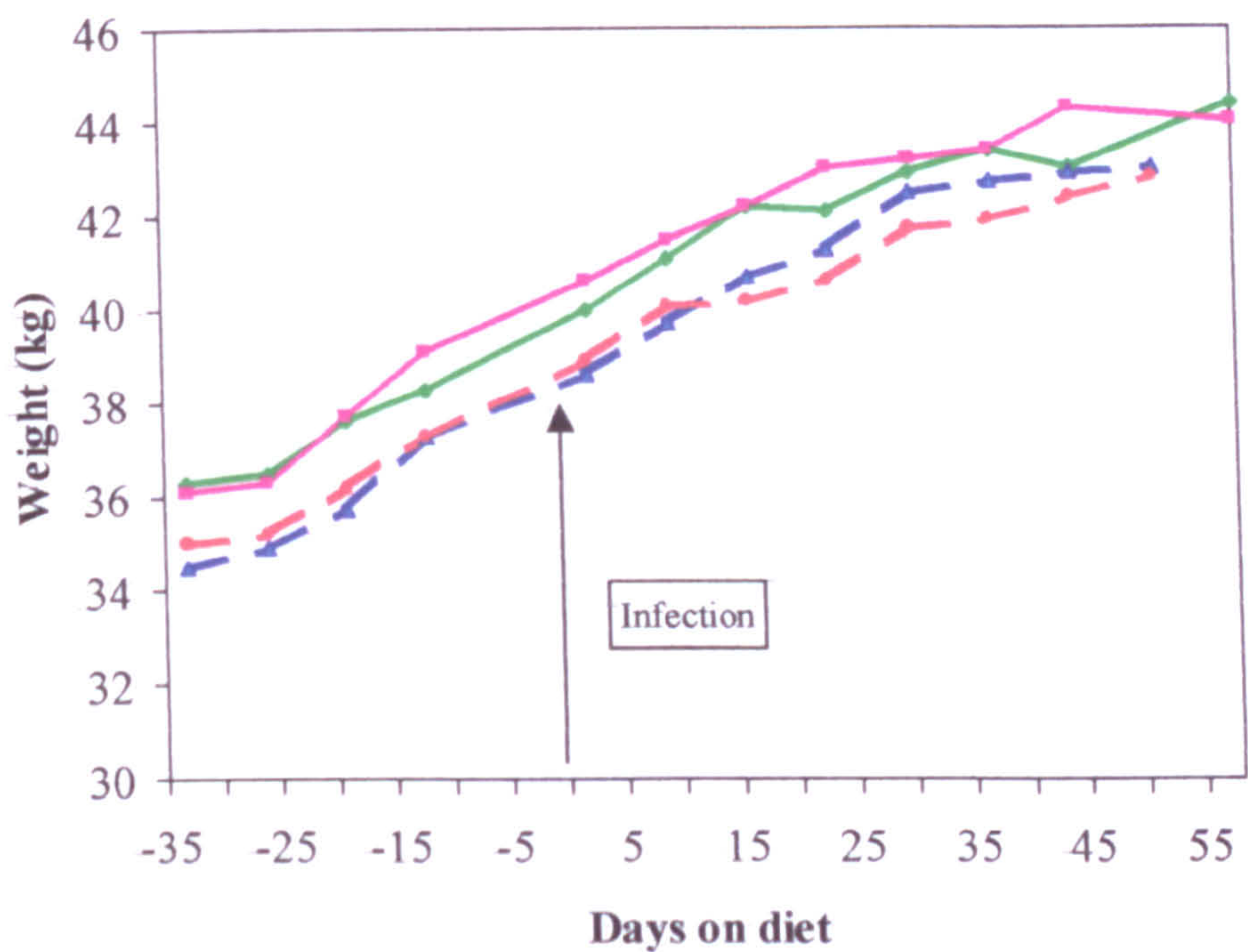
### 4.3.2 Faecal analysis

#### 4.3.2.1 *Faecal egg counts*

Faecal egg counts taken prior to the infection period confirm show that all lambs remained parasite free prior to the infection trial (data not shown). Total faecal output and dry matter content were not measured during the trial, hence faecal egg counts were calculated on a gram per fresh faecal basis (EPG). The profile of egg output during the infection period is shown in Figure 4.2. Eggs were first observed in the faeces 18 days p.i. with peak egg production at approximately 32 days p.i., which declined thereafter. Egg output followed a quadratic pattern over time ( $p<0.001$ ). Peak egg output was greatest in male control-fed lambs (being 2-fold greater than other groups at approximately 2400 EPG) and accompanied by the most rapid rate of decline in egg counts (gradient of decline  $\sim 55$  eggs/d). Control-fed females produced the next largest peak egg output (approximately 1100 EPG, gradient of decline  $\sim 35$  eggs/d). Results from the tannin-fed lambs were similar in males and females and markedly lower than controls, the rate of decline being approximately 15 eggs/d.



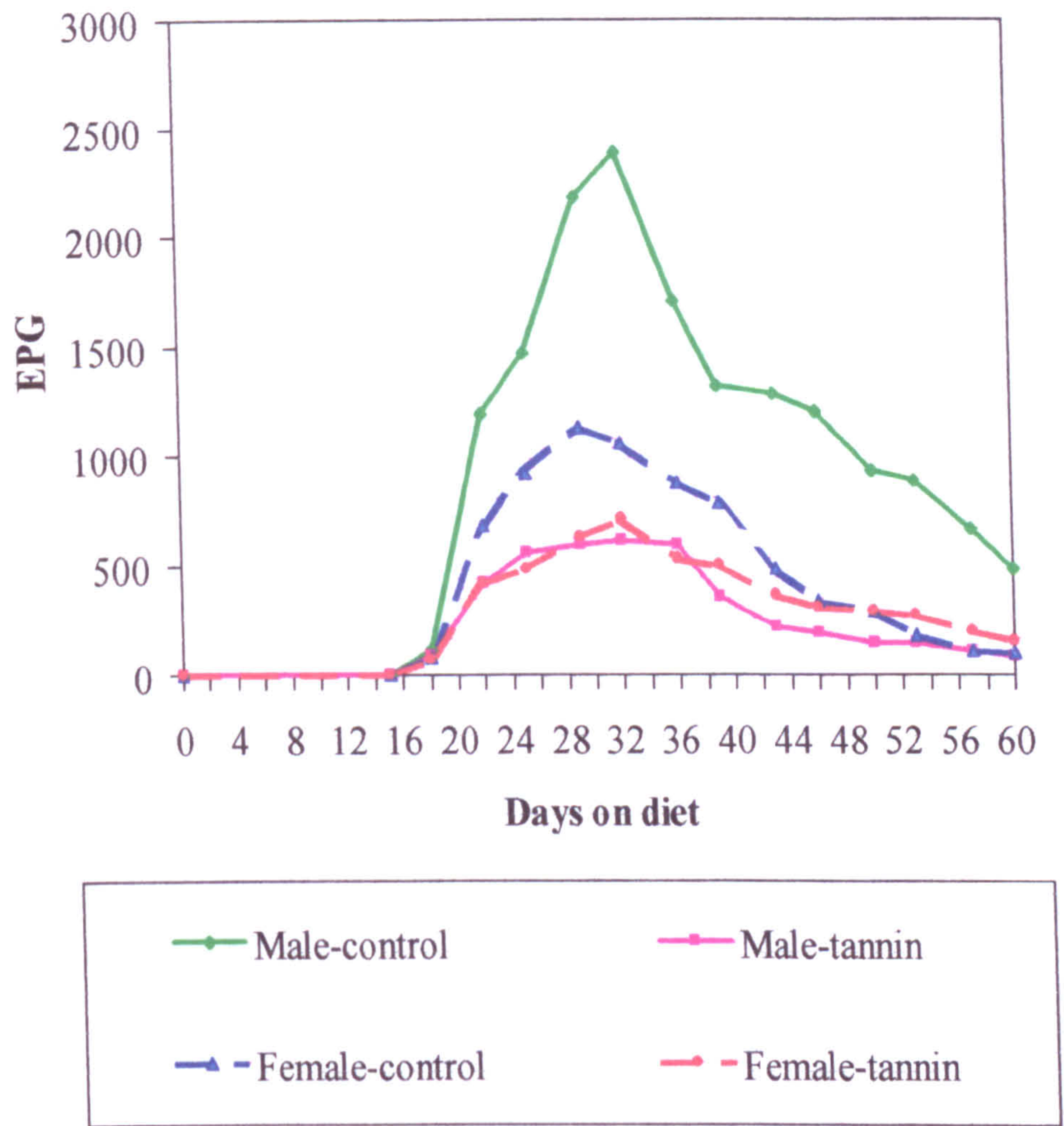
**Figure 4.1** Effect of dietary quebracho tannin on growth and daily liveweight gains of *T. colubriformis* infected lambs



Daily liveweight gains  
(12 observations from the introduction of tannin diets to termination)  
Male-control 95.3 g/d; Male-tannin 98.7 g/d;  
Female-control 112.8 g/d; Female-tannin 98.2 g/d.  
  
n = 5 lambs/experimental group, except female-tannin  
where n = 4 lambs



**Figure 4.2** Effect of dietary quebracho tannin on the number of eggs (EPG) of *T. colubriformis* infected lambs



n = 5 lambs/experimental group, except female-tannin where n = 4 lambs



Faecal egg counts fell to low levels (below 500 EPG) in male tannin-fed and both female groups by approximately week 7 p.i., EPG in male control-fed lambs fell to around 500 EPG by the end of the experiment (60 days p.i.). The decline of faecal egg counts in female tannin-fed lambs was less rapid than control-fed females resulting in terminal egg counts being marginally greater than the control-fed females (142 vs. 66 EPG). Faecal consistency changed during the infection period; from approximately week 4 after infection onwards, faeces from several lambs tended to have a loose wet structure. Mean eggs per gram of fresh faeces are displayed in Table 4.1. Diet had a significant effect ( $p=0.030$ ), control-fed animals excreting more than double the number of nematode eggs than tannin-fed animals. While no significant difference was seen between sexes, female lambs were approximately 40% less susceptible to *T. colubriformis* infection. This was predominantly due to the very low infection seen in the female control-fed lambs compared to male control-fed lambs. Tannin-fed male and female lambs excreted similar numbers of eggs in their faeces.

#### *4.3.2.2 Total faecal output*

Mean total faecal and urine output from four 24h collection periods are shown in Table 4.1. The inclusion of dietary tannin in the diets of the male lambs significantly increased total daily faecal output ( $p<0.05$ ). This was not observed in the female tannin-fed lambs.

### **4.3.3 Blood analysis**

#### *4.3.3.1 Eosinophils*

The concentrations of eosinophils circulating in peripheral blood are shown in Figure 4.3. Eosinophil concentration remained low until approximately 35 days p.i. when a rapid rise was observed in all animals and continued until slaughter. Eosinophil concentrations were meaned from when eosinophils began to rise (day 21 p.i.) to the



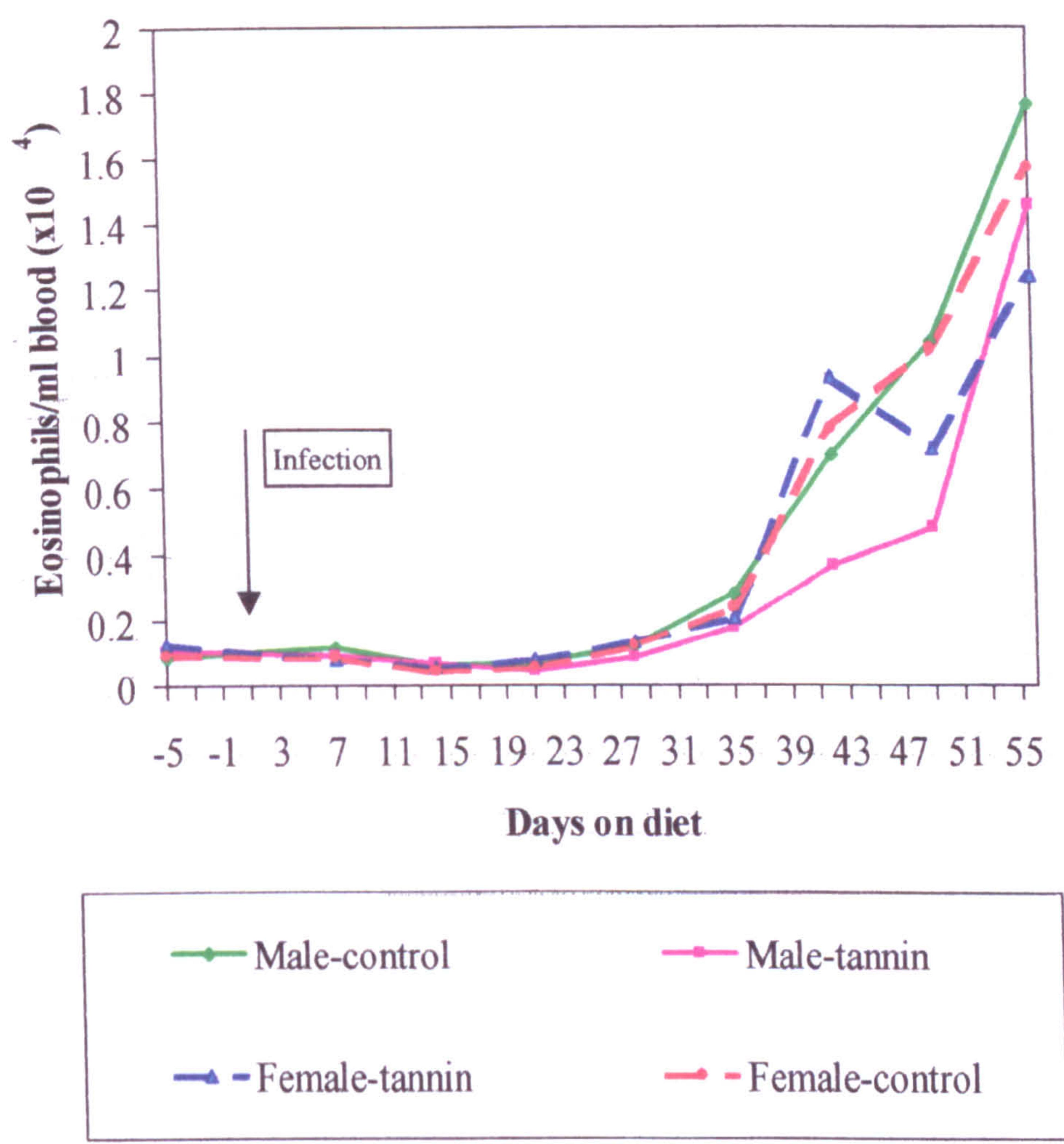
**Table 4. 1** Effect of dietary quebracho tannin on mean faecal eggs per gram (EPG) passed throughout the infection period and mean faecal output during a 4 day collection period while lambs were housed in metabolism crates

| Group        | Male-control | Male-tannin | Female-control | Female-tannin | s.e.d.<br>(15 d.f.) | Statistical significance of difference |       |            |
|--------------|--------------|-------------|----------------|---------------|---------------------|--|-------|------------|
|              |              |             |                |               |                     | diet                                   | sex   | diet x sex |
| EPG          | 1158         | 298         | 505            | 361           | 269.8               | 0.030                                  | 0.181 | 0.109      |
| faeces (g/d) | 1208         | 1582        | 1544           | 1568          | 134.4               | 0.054                                  | 0.111 | 0.085      |
| urine (g/d)  | 1452         | 1177        | 1257           | 1556          | 279.6               | 0.949                                  | 0.648 | 0.167      |

EPG figures are presented as the average of 13 time points once egg were present in the faeces



**Figure 4.3** Effect of dietary quebracho tannin on the number of eosinophils circulating in peripheral blood of *T. colubriformis* infected lambs



n = 5 lambs/experimental group, except female-tannin where n = 4 lambs



end of the trial (day 60 p.i.). No statistically significant differences in mean eosinophil concentration were seen between the four treatment groups.

#### 4.3.3.2 Amino acid profile

All animals were cannulated at two time points during the trial to allow serial blood samples to be collected over a 48h period to reduce any diurnal and feeding effects on amino acid profiles circulating in peripheral blood. No significant sex differences were observed and hence the data is displayed for diet and time interactions only (Table 4.2).

The only significant effect of diet was seen for cystine, where the concentration was reduced by the inclusion of dietary eQT. *T. colubriformis* infection (comparing time point 1 with time point 2) reduced asparagine, cystine, glutamine, glycine, histidine, proline, threonine, tryptophan and tyrosine; phenylalanine concentration fell only in the control-fed lambs. An elevation in alanine and lysine concentration with infection was observed, the elevation in lysine was only apparent in tannin-fed lambs.

The amino acid, arginine, was affected by a diet x time interaction, the concentration falling with time in control-fed lambs, but increasing in tannin-fed lambs. Aspartic acid concentrations significantly increased after infection in control-fed lambs and remained unaffected in tannin-fed lambs. Citrulline concentrations decreased after infection for both dietary treatments, significant increase in control-fed lambs only. Glutamic acid concentrations significantly increased following infection, being greater in control-fed lambs and ornithine concentrations where a non-significant decline was seen following infection in control-fed lambs but non-significantly increased in tannin-fed lambs after infection.

The total circulating concentrations for amino acids was not affected by diet but concentrations tended to decline due to infection ( $p=0.061$ ). Tryptophan was either found at very low concentrations in the plasma or could not be identified in the chromatograms and has been omitted from the data presented for the sum of the amino acids.

**Table 4.2** Amino acid concentrations circulating in peripheral blood (nMole/ml), time period 1 taken prior to *T. colubriformis* infection once the lambs were acclimatised to the experimental diets (days -5 to -3 p.i.) and time period 2 taken during the period of worm expulsion (days 44 to 46 p.i.)

| Group         | Control |       | Tannin |       | s.e.d.<br>30 d.f. | Statistical significance of difference |                     |
|---------------|---------|-------|--------|-------|-------------------|--|---------------------|
|               | 1       | 2     | 1      | 2     |                   | diet                                   | time<br>diet x time |
| Time period   |         |       |        |       |                   |  |                     |
| Alanine       | 164.7   | 184.2 | 165.2  | 180.4 | 11.72             | 0.865                                  | 0.015               |
| Arginine      | 111.5   | 103.5 | 117.5  | 123.7 | 7.52              | 0.059                                  | 0.813               |
| Asparagine    | 31.58   | 27.10 | 32.75  | 27.49 | 2.731             | 0.707                                  | 0.015               |
| Aspartic acid | 12.34   | 20.39 | 13.17  | 13.34 | 2.019             | 0.037                                  | 0.013               |
| Citrulline    | 161.8   | 100.4 | 158.8  | 132.6 | 15.58             | 0.288                                  | <0.001              |
| Cystine       | 12.47   | 10.83 | 10.16  | 7.18  | 1.008             | 0.002                                  | 0.002               |
| Hisidine      | 62.8    | 55.8  | 62.5   | 55.5  | 3.83              | 0.908                                  | 0.018               |
| Glutamine     | 352.0   | 282.5 | 332.4  | 271.7 | 18.25             | 0.331                                  | <0.001              |
| Glutamic acid | 75.3    | 169.4 | 77.7   | 129.7 | 11.03             | 0.035                                  | <0.001              |
| Glycine       | 500.7   | 456.3 | 492.9  | 423.5 | 24.57             | 0.324                                  | <0.001              |



| Group              | Control |       | Tannin |       | s.e.d.<br>30 d.f.  | Statistical significance of difference |        |             |
|--------------------|---------|-------|--------|-------|--------------------|--|--------|-------------|
|                    | 1       | 2     | 1      | 2     |                    | diet                                   | Time   | diet x time |
| Time period        |         |       |        |       |                    |  |        |             |
| Isoleucine         | 130.2   | 135.1 | 122.9  | 130.3 | 7.87               | 0.398                                  | 0.104  | 0.726       |
| Leucine            | 214.9   | 215.4 | 203.1  | 219.4 | 14.30              | 0.771                                  | 0.139  | 0.165       |
| Lysine             | 109.9   | 112.2 | 106.9  | 120.7 | 8.04               | 0.704                                  | 0.040  | 0.128       |
| Methionine         | 19.45   | 18.81 | 17.42  | 18.12 | 1.359              | 0.219                                  | 0.977  | 0.437       |
| Ornithine          | 173.7   | 165.5 | 171.4  | 188.7 | 14.64              | 0.461                                  | 0.360  | 0.018       |
| Phenylalanine      | 79.4    | 69.2  | 74.7   | 75.5  | 5.82               | 0.871                                  | 0.115  | 0.069       |
| Proline            | 132.0   | 115.1 | 117.5  | 107.8 | 9.35               | 0.163                                  | 0.029  | 0.527       |
| Serine             | 128.0   | 116.6 | 135.3  | 129.5 | 12.24              | 0.370                                  | 0.138  | 0.612       |
| Threonine          | 235.7   | 193.9 | 238.1  | 207.0 | 20.90              | 0.691                                  | <0.001 | 0.537       |
| Tryptophan         | 82.6    | 60.7  | 81.9   | 51.1  | 11.31              | 0.442                                  | 0.024  | 0.640       |
| Tyrosine           | 99.1    | 80.2  | 93.9   | 82.6  | 6.34               | 0.798                                  | <0.001 | 0.273       |
| Valine             | 408.8   | 381.7 | 379.5  | 380.8 | 27.33              | 0.567                                  | 0.163  | 0.126       |
| Total <sup>1</sup> | 3215    | 3014  | 3124   | 3024  | 157.7 <sup>2</sup> | 0.771                                  | 0.060  | 0.515       |

<sup>1</sup> Total excludes tryptophan

<sup>2</sup> 26 d.f.

#### 4.3.4 Worm burden and eggs *in utero*

Subsamples of the number of *T. colubriformis* worms recovered from the small intestine of each lamb were counted and then multiplied to give the total worm burden (Table 4.3). The worm burden residing in the small intestine at slaughter from female lambs was not reduced by dietary tannin compared to control-fed females ( $p>0.1$ ). These data reflect terminal faecal egg counts where tannin-fed females had a final mean EPG of 142 compared to 66 for control-fed females. Male lambs fed the tannin diet had significantly fewer worms inhabiting their gut compared to control-fed males ( $p<0.05$ ). A subsample of worms were sexed and the number of egg *in utero* of female worms counted. No significant differences were seen between groups (Table 4.3).



**Table 4. 3** Effect of dietary quebracho tannin on the *T. colubriformis* worm burdens recovered from lambs. Eggs *in utero* were counted from randomly chosen female worms from each lamb.

| Group                | Male-control | Male-tannin | Female-control | Female-tannin | s.e.d.<br>(15 d.f.) | Statistical significance of difference |       |            |
|----------------------|--------------|-------------|----------------|---------------|---------------------|--|-------|------------|
|                      |              |             |                |               |                     | diet                                   | sex   | diet x sex |
| Worm burden          | 29656        | 7688        | 14112          | 21320         | 9491.7              | 0.289                                  | 0.889 | 0.046      |
| Eggs <i>in utero</i> | 2.89         | 0.38        | 1.37           | 1.91          | 1.294               | 0.301                                  | 1.000 | 0.118      |

#### 4.4 PRELIMINARY DISCUSSION

The aim of this sheep study was to establish if the model condensed tannin, quebracho tannin, could affect the persistence of *T. colubriformis* in the small intestine of castrate male and female lambs maintained on a feed intake-restricted diet.

The inclusion of quebracho tannin in an energy-restricted diet did not improve the growth rate of lambs. The persistence of infection was monitored through faecal egg counts. Dietary quebracho tannin inclusion reduced EPG, with a diet x sex interaction being observed. Control-fed male lambs were more susceptible to *T. colubriformis* infection than female-control fed lambs. The inclusion of quebracho tannin significantly reduced EPG in male lambs. A difference in egg counts between control-fed male and female lambs was apparent, no difference between sexes was observed in the tannin-fed animals. Reports vary as to whether males are more susceptible than females to parasitic infection, data from two flocks reported by Eady *et al.* (1996) show significant differences where ewes had lower faecal egg counts than wethers, while Woolaston & Piper (1996) reported no consistent differences between ewe and ram lambs run together over a number of years. One possible explanation for the increased susceptibility of male lambs to *T. colubriformis* infection in this trial could be the development of sexual maturity. At slaughter, two male lambs used in this trial were shown to have remained entire during the trial. Male sheep have been reported to be more susceptible than females to a number of different challenge helminth infections given around or after puberty e.g. *Oesophagostomum columbianum* (Bawden, 1969) and *T. colubriformis* (Windon & Dineen, 1981). In a review by Barger (1993) consistent effects of host sex on susceptibility to infection were not seen in younger, possibly pre-pubertal lambs. Mechanisms for these differences have been elucidated from rodent studies reviewed by Barger (1993) using gonadectomy and/or hormone replacement. Testicular hormones decreased resistance and ovarian hormones increased resistance to nematode parasitism. Physiological concentrations of oestrogens in females have been shown to stimulate immune responses, while androgens have the opposite



effect. Circulating concentrations of antibodies and major immunoglobulin classes are higher and more sustained in females than in males. This heightened immune response of females may reduce their susceptibility to parasitic infection.

Faecal output was only estimated over a 4 day period at the end of the trial (week 8 p.i. for male lambs and week 9 p.i. for female lambs). An increase of 30% in daily faecal output was observed from the male tannin-fed lambs and would significantly alter the daily faecal egg output by a dilution effect. Faecal output measured at one time point during a 9 week infection study can only give an indication of how increased faecal output could affect faecal egg counts recorded as EPG. Assuming male tannin-fed lambs excreted 30% more faeces than control-fed males, the difference in total daily faecal egg output would be less than 3-fold, while EPG figures show that tannin-fed males excrete almost 4-fold greater EPG compared to control-fed males. Faecal output was not increased from female tannin-fed lambs compared to control-fed females.

Faecal egg counts have been shown to have a good relationship with worm burdens for sheep and goats (Cabaret *et al.*, 1998), therefore quebracho tannin may have depressed EPG by reducing the number of worms present in the small intestine. Worm burdens recovered at slaughter indicate that tannin-fed male lambs had significantly smaller numbers of *T. colubriformis* residing in the small intestine compared to control-fed males.

The blood parameters measured during this study did not produce any significant differences. Eosinophils were not elevated by the inclusion of quebracho tannin in the diet, thus quebracho tannin does not appear to promote an inflammatory response which is reflected through peripheral eosinophil concentrations. Amino acid concentrations in peripheral blood were analysed to determine whether dietary quebracho tannin altered amino acid concentrations and whether there was an increased demand for these amino acids during worm elimination. Low levels of condensed tannins, similar to that used in this trial, consumed by young sheep have

been shown to 'protect' sulphur amino acids during digestion and elevate the circulating concentrations of these amino acids in the blood, possibly influencing whole body metabolism (Lee *et al.*, 1995). Sulphur amino acids are a component of mucus and leukotrienes which are both components known to prevent worm establishment and promote worm expulsion (Lindsay *et al.*, 1980; Kimambo *et al.*, 1988; McClure *et al.*, 1992). If dietary quebracho tannin could increase the circulating concentrations of sulphur amino acids in the blood these could then provide important components involved in resistance and resilience mechanisms against small intestinal nematode infection. To accurately measure the cystine and cysteine concentrations, plasma samples need to be deproteinised shortly after collection since storage promotes the binding to these amino acids to protein that can be subsequently lost during deproteinisation (Malloy *et al.*, 1981). Unfortunately the plasma samples from this study were deep-frozen for several months before deproteinisation and concentrations of sulphur amino acids were low. Threonine and histidine are two further essential amino acids that are components of mucus. In the present study peripheral blood concentrations of these amino acids were depressed during the period of worm expulsion. Feeding dietary tannins has been reported to increase concentrations of cysteine, methionine and glutathione in protein-free whole blood or plasma (Lee *et al.*, 1995) an increase only for cystine was reported by Wang *et al.* (1994). Waghorn *et al.* (1987b) observed the concentrations of amino acids (essential and non-essential) were higher in condensed tannin-fed sheep in omasal, abomasal and ileal digesta, the net absorption of amino acids from the intestine were not significantly different for tannin or polyethylene glycol (PEG)-dosed sheep. Likewise, PEG did not affect amino acids circulating in peripheral blood. When time point 1 was considered in the present study (before nematode infection) the only effect of dietary quebracho tannin seen was the reduction of cystine ( $p=0.047$ ) and proline ( $p=0.054$ ). The decline in cystine may be due to hypersecretion of mucus due to possible gastrointestinal breakdown caused by the presence of quebracho tannin in the digesta.

In summary, dietary quebracho tannin reduced *T. colubriformis* infection in lambs. Male lambs appeared to be more susceptible to infection than female lambs. Animals fed dietary tannins excrete greater quantities of faecal matter (observed using the



rodent model and in the present chapter). To confirm the reduction in faecal egg counts from tannin-fed lambs, daily faecal output needs to be measured when egg counts are recorded to provide a total daily faecal egg output. Thus, a reduction in parasite infection after feeding quebracho tannin could then be confirmed. Chapter 5 reports a more complicated sheep study attempting to confirm observations in the present study. In addition, in this study the effect of providing dietary quebracho tannin once *T. colubriformis* infection has established in the small intestine and the interaction of increasing dietary protein concentration were also investigated.

## CHAPTER 5

### EFFECT OF DIETARY QUEBRACHO TANNIN AND PROTEIN CONCENTRATION ON NEMATODE INFECTION IN LAMBS (SHEEP STUDY 2)

#### 5.1 INTRODUCTION

Animals maintained on a low plane of nutrition are more susceptible to parasitic infection compared to their well-fed counterparts. Increasing dietary protein supply enables infected animals to overcome some of the debilitating effects associated with parasitic infection (Poppi, *et al.*, 1986; Abbott *et al.*, 1988; Bown *et al.*, 1991b; Kyriazakis *et al.*, 1994; van Houtert *et al.*, 1995a,b, 1996). The development of resistance is also believed to be dependent on protein status (Bown *et al.*, 1991b; Kambara *et al.*, 1993). Results from sheep study 1 (chapter 4) indicate that lambs fed diets containing quebracho tannin (eQT) had reduced numbers of *Trichostrongylus colubriformis* eggs in the faeces, possibly indicating a reduced level of parasite infection. These lambs were maintained on a low plane of nutrition through feeding a feed intake-restricted diet. The objective of the second sheep study was to investigate the inclusion of eQT in low and high protein diets fed to *T. colubriformis* infected lambs. High protein diets appear to have little effect on initial establishment in naive sheep (as reviewed by Coop & Holmes, 1996). Thus all lambs were maintained on a low protein diet until *T. colubriformis* infections had established in the small intestine. At this time point changes were made to increase the dietary protein concentration and/or to include dietary eQT. The immune status of the animals was assessed through haematological parameters including the IgG response to a foreign protein, ovalbumin and the response to *T. colubriformis* monitored by faecal egg output. The study was concluded by administering a challenge infection and recovery of the worm burden 10 days later.



## **5.2 MATERIALS AND METHODS**

### **5.2.1 Animals and housing**

Thirty-six castrate male lambs (Charolais X mule) were purchased from the University of Nottingham farm at weaning, approximately 6 weeks of age. The lambs were born over a two week period, drenched with Cydectin (Cyanamid, Crawley, W. Sussex) at weaning, and housed indoors 2 weeks later. Cydectin containing moxidectin, rapidly kills all important sheep nematodes and has a persistent activity preventing re-infection for the subsequent five week period. Animals were therefore considered to be free from parasites at housing, which was confirmed by random faecal egg counts (section 2.3.2.2), and remained so until the infection period began (data not shown).

### **5.2.2 Pre-trial period**

The lambs were fed pelleted dried grassmeal for 6 weeks following housing. Feed offered was increased only up to 1 kg/d to maintain the lambs on a low plane of nutrition prior to the introduction of experimental diets. One month before the infection period began, experimental diets were introduced. Lambs were randomly allocated into one of six groups (section 5.2.4.1) and fed the appropriate pelleted experimental diet at 4% bodyweight. Animals were weighed twice weekly using a weigh crate connected to a digital balance [Tru-test AG500, Tru-Test Distributors Ltd., Auckland, New Zealand] and feed allowance calculated. The weigh crate was routinely calibrated before use with a standard weight of 20 kg. Any feed refusals were collected prior to the morning feed and weighed.

### 5.2.3 Experimental diets

High and low protein diets were formulated to have a similar estimated metabolisable energy value of approximately 10.5 MJ/kg diet. This was obtained using the ingredients listed in table 5.1, estimated values were taken from Agricultural and Food Research Council figures (1993). Both diets contained the same ingredients with the addition of fishmeal as a high protein source in the high protein diet. The low protein diet (L) was designed to mimic a poor quality diet with an estimated crude protein content of 97 g/kg. The high protein diet (H), 222 g CP/kg had a surplus of high quality protein and was not thought to compromise the animals' growth pattern. During the trial the lambs were fed either diet L (97 g/kg) or H (222 g/kg) with or without eQT [type ATO, Hodgesons Chemicals Ltd, Beverly, Hull]. The diets were offered at 4% bodyweight (Kyriazakis *et al.*, 1996a) once daily. Where appropriate, eQT was incorporated into the diet, prior to mixing and pelleting, at a level of 50 g eQT/kg. The tannin was assumed to be nutritionally inert, consequently lambs fed the tannin containing diets were fed proportionally (0.05) more than control-fed lambs to maintain approximately constant nutrient presentation. All diets fed during the infection period (days 0 to 71 p.i.) contained 0.5 g chromic oxide ( $\text{Cr}_2\text{O}_3$ )/kg feed [Fisher Scientific UK, Loughborough, Leicestershire] as a faecal output marker (Kotb and Luckey, 1972).

### 5.2.4 Trial period

#### 5.2.4.1 Group allocation

The lambs were maintained on a pelleted diet of dried grass until 19 weeks of age when the experimental period began (mean liveweight  $32.6 \pm 3.9$  kg). Lambs were randomly assigned to one of 6 groups and fed the diet L either without (groups 1, 3, 4, 5 & 6) or with eQT (group 2) for 31 days prior to the infection period. At 23 weeks of age, groups 1-5 were infected daily with 3000 infective stage ( $L_3$ ) *T.*



**Table 5. 1** Diet composition (g/kg fresh weight)

|  | Low Protein (L) | High Protein (H) |
|--|-----------------|------------------|
| Dried grass                            | 50.0            | 397.7            |
| Wheat straw + NaOH                     | 77.1            | 47.4             |
| Molassed beet                          | 350.0           | 72.0             |
| Barley                                 | 500.0           | 290.0            |
| Fishmeal                               | 0.0             | 170.0            |
| Vits/mins <sup>1</sup>                 | 22.9            | 22.9             |
| Estimated Crude protein                | 97              | 222              |
| Estimated Metabolisable energy (MJ/kg) | 10.5            | 10.5             |

For diets containing extracted quebracho tannin (eQT), 50 g eQT/kg was added to the diets prior to pelleting to give the corresponding low protein + eQT and high protein + eQT.

Estimated crude protein and metabolisable energy are based on figures reported by the Agricultural and Food Research Council (1993).

|                        |            |        |                |
|------------------------|------------|--------|----------------|
| Vits/mins <sup>1</sup> |            |        |                |
| Ammonium Chloride      | 10%        | Vit A  | 320000 i.u./kg |
| Calcium                | 22%        | Vit D3 | 80000 i.u./kg  |
| Phosphorus P           | 2%         | Vit E  | 800 i.u./kg    |
| Sodium                 | 9.8%       |        |                |
| Cobalt                 | 80 mg/kg   |        |                |
| Iodine                 | 200 mg/kg  |        |                |
| Manganese              | 2000 mg/kg |        |                |
| Selenium               | 8 mg/kg    |        |                |
| Zinc                   | 2000 mg/kg |        |                |

*colubriformis* larvae for 10 weeks. Group 6 remained as uninfected controls. Following nematode establishment, as judged by the presence of eggs in the faeces (23 days post infection (p.i.)), diets were abruptly changed in groups 3, 4 and 5 (Table 5.2) to investigate the effect of increasing dietary protein and/or eQT inclusion on the ability of the animals to mount an immune response to infection. Group 2 lambs (LeQT prior and p.i.) were included in the trial to ascertain whether tannin was required in the diet prior to infection.

#### 5.2.4.2. Nematode infection and diet changes

One month following the introduction of the experimental diets lambs in groups 1-5 were exposed to a trickle infection of *T. colubriformis* larvae (L<sub>3</sub>). The animals were infected *per os* on 5 days/week with doses of 6000, 3000, 3000, 3000 and 6000 L<sub>3</sub> as stated in section 2.2. Lambs assigned to group 6 remained uninfected throughout the trickle infection and acted as an uninfected control group.

#### 5.2.4.3 Faecal grab sampling

Faecal samples (approximately 15 g) were obtained by sampling directly from the rectum at regular intervals for determination of egg counts and chromium content. Faecal egg counts were monitored twice weekly or every second day around peak egg output. The number of eggs per gram of faeces (EPG) was determined using the method described in section 2.2.2. Eggs recovered were expressed on a faecal dry weight basis. Total daily egg output was calculated using estimations of daily faecal output from chromic oxide concentration in the remaining grab sample. Chromium content was determined in the remaining samples (taken for egg counting) after acid hydrolysis and atomic absorption spectrometry using a nitrous oxide-acetylene flame (Siddons *et al.*, 1985) (section 2.7). Total faecal collection, using dungbags, was undertaken on 2 animals from each infected group (groups 1-5) during the period of faecal sampling for egg counts to validate the estimates of total faecal output calculated from the recovery of chromium in the faeces.



Table 5. 2 Dietary groupings of lambs

| Group                        | 1     | 2           | 3     | 4          | 5         | 6            |
|------------------------------|-------|-------------|-------|------------|-----------|--------------|
|                              | (L-L) | (LeQT-LeQT) | (L-H) | (L-HeQT)   | (L-LeQT)  | (uninfected) |
| Initial diet                 | Low   | Low + eQT   | Low   | Low        | Low       | Low          |
| (age 19-23 weeks)            |       |             |       |            |           |              |
| Diet after nematode          | Low   | Low + eQT   | High  | High + eQT | Low + eQT | Low          |
| Establishment (23 days p.i.) |       |             |       |            |           |              |

Low = Low protein - 97 g/kg

High = High protein – 222 g/kg

+ eQT, quebracho tannin added at 50 g/kg

#### *5.2.4.3 Blood sampling*

Plasma samples were collected post-feeding at approximately weekly intervals by jugular venipuncture (section 2.4.1) for the determination of blood eosinophil concentrations (section 2.4.2) and plasma concentration of total protein and albumin (sections 2.4.4 & 2.4.5). Globulin concentrations were calculated by difference.

#### *5.3.4.4 Immune responsiveness*

To estimate immune responsiveness, each animal was given a subcutaneous injection of ovalbumin [Grade V, Sigma Chemicals Co., Dorset]. The animals were first injected 50 days p.i., with a booster injection administered 10 days later. The sheep were immunised with 1 mg of ovalbumin and 500 mg of the adjuvant Quil-A [Superfos Biosector a/s, Frydenlundsvej 30, DK-Vedbaek, Denmark] given in 2 ml of Dulbecco's phosphate buffered saline [Sigma Chemical Co., Dorset]. The antigen was subcutaneously injected at 4 individual sites (0.5 ml/site) along one side of the back of the animals, the second booster injections were administered on the other side of the back. Serum was collected 6 days after the booster injection (section 2.4.1) and stored at -40 °C prior to the estimation of immune responsiveness of the animals by the level of IgG response to injected ovalbumin, analysed using a standard enzyme linked immunosorbant assay (ELISA) as described in section 2.5.1.

#### *5.2.4.5 Challenge infection*

The trickle infection was continued for 10 weeks. At the end of the 10 week period all 36 animals (groups 1-6) were drenched with anthelmintic [Parafend, Oxfendazole 2.265 %, Norbrook Laboratories, Worcestershire, UK] to remove any remaining worm burden from the small intestine. All lambs were fed diet L. Approximately one week later all lambs were given a challenge infection of 15 000 L<sub>3</sub> *T. colubriformis* suspended in 10 ml distilled water. Ten days later the animals were slaughtered.



### **5.2.5 Slaughter protocol**

The animals were humanely slaughtered by conventional slaughterhouse techniques (stunned and exsanguinated). A blood sample and faecal sample were collected for analysis.

The entire intestinal tract was removed following ligation as described in section 2.3.3, and the gut processed for worm recovery. In addition the omental fat surrounding foregut (reticulo-rumen, omasum and abomasum) was removed and weighed. The perirenal fat surrounding the kidneys was removed and the weight recorded. The liver, heart and spleen were removed from the carcass and the empty warm carcass weight was recorded.

The worms recovered from the small intestine and fixed using formalin were stained with helminthological iodide and examined with a low powered dissecting microscope as described in section 2.3.3.2.

### **5.2.6 Statistical analysis**

Data were analysed as a six-treatment completely randomised experiment, with six replicate animals in each treatment, using one-way analysis of variance [Genstat 5, Release 4.1; Lawes Agricultural Trust, Rothamsted). Repeated measures were used to analyse changes with time within-treatments. The Greenhouse-Geisser epsilon factor obtained from repeated measures analysis was used to adjust the degrees of freedom for time x diet interaction (Winer *et al.*, 1991). Data were further partitioned into linear, quadratic and cubic trends.

Variation between the six treatments was further partitioned for the effect of parasitism (uninfected lambs (group 6) versus infected animals (groups 1-5)) and a 2

x 2 factorial was used to compare dietary protein content (high and low protein)  $\pm$  eQT. T-tests using the pooled standard error of difference between means were used to compare the effect including eQT before and after nematode infection (group 2) with those changed from diet L to LeQT once eggs were passed in the faeces (group 5). Data were blocked for sheep. Differences were assumed to be significantly different at  $p \leq 0.05$ .



## 5.3 RESULTS

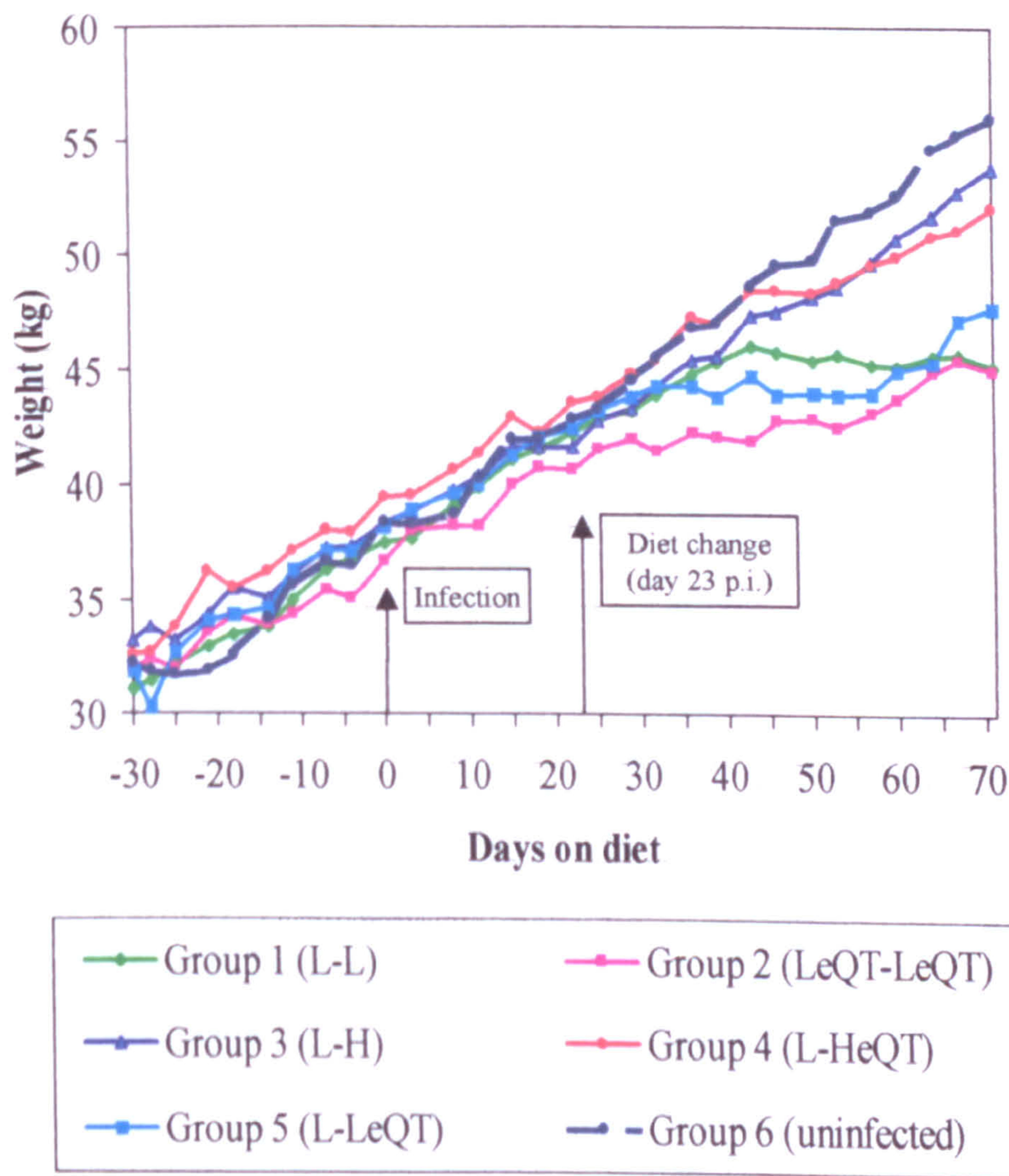
### 5.3.1 Liveweight and feed intake

The mean growth rates of lambs in each dietary group over the experimental period are shown in Figure 5.1. There were no statistically significant differences ( $p>0.2$ ) between the mean liveweights of the animals in the six treatment groups between day 0 ( $38.1 \pm 2.9$  kg) and day 23 ( $42.9 \pm 3.1$  kg). Infection tended to reduce mean liveweight compared to the uninfected controls (group 6) over the period from diet change until the end of the experiment (day 23 p.i. to day 71 p.i.). Mean liveweights at the end of the trial were highest in uninfected controls (56 kg liveweight) while infected lambs maintained on the LeQT (groups 1, 2 & 5) weighed 45.1, 45.0 & 47.7 kg respectively ( $p<0.05$ , s.e.d. 3.8, d.f. 30). Increasing dietary protein content from 97 to 222 g/kg  $\pm$  eQT (groups 3 & 4) increased mean liveweight of infected animals with the final liveweights of the high protein  $\pm$  eQT fed lambs (groups 3 & 4) being similar to uninfected controls (53.9 & 52.1 kg for groups 3 & 4 respectively compared with 56 kg for group 6,  $p>0.2$ , s.e.d. 3.8, d.f. 30). The inclusion of eQT in the diet did not alter the mean liveweights of the animals fed the same protein diet during the period from diet change to the end of the experiment.

Infected animals all showed some degree of inappetence during the trickle infection but no further reductions in intake were observed in lambs fed tannin-containing diets. The mean daily feed intake (Table 5.3) of the lambs was similar in all groups pre-infection ( $p>0.1$ ). Feed refusals were first observed once the nematodes had established in the small intestine, after which, infected lambs maintained on the low protein diets  $\pm$  eQT (groups 1, 2 & 5) consumed less feed compared to those fed high protein diets  $\pm$  eQT (groups 3 & 4,  $p<0.05$ ). Lambs fed the high protein diet  $\pm$  eQT maintained a similar feed intake to the uninfected controls.



**Figure 5.1** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or the inclusion of dietary quebracho tannin) on the growth of lambs until anthelmintic treatment (day 75 p.i.)





**Table 5. 3 Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or dietary quebracho tannin) on mean daily feed intake of lambs in each dietary group (g feed/kg bodyweight)**

| Group             | 1<br>(L-L)          | 2<br>(LeQT-LeQT)  | 3<br>(L-H)          | 4<br>(L-HeQT)         | 5<br>(L-LeQT)       | 6<br>(uninfected) | s.e.d.<br>(30 d.f.) |
|-------------------|---------------------|-------------------|---------------------|-----------------------|---------------------|-------------------|---------------------|
| Pre-infection     | 38.3                | 38.8              | 38.1                | 38.7                  | 38.5                | 37.0              | 1.99                |
| Days 0 - 23 p.i.  | 38.7                | 38.4              | 39.5                | 38.4                  | 38.7                | 38.1              | 0.52                |
| Days 23 - 71 p.i. | 31.3 <sup>a,b</sup> | 30.2 <sup>a</sup> | 36.4 <sup>b,d</sup> | 36.0 <sup>b,c,d</sup> | 31.1 <sup>a,c</sup> | 38.1 <sup>d</sup> | 2.53                |

<sup>a,b,c,d</sup> means with different superscripts within a row are significantly different (p<0.05)

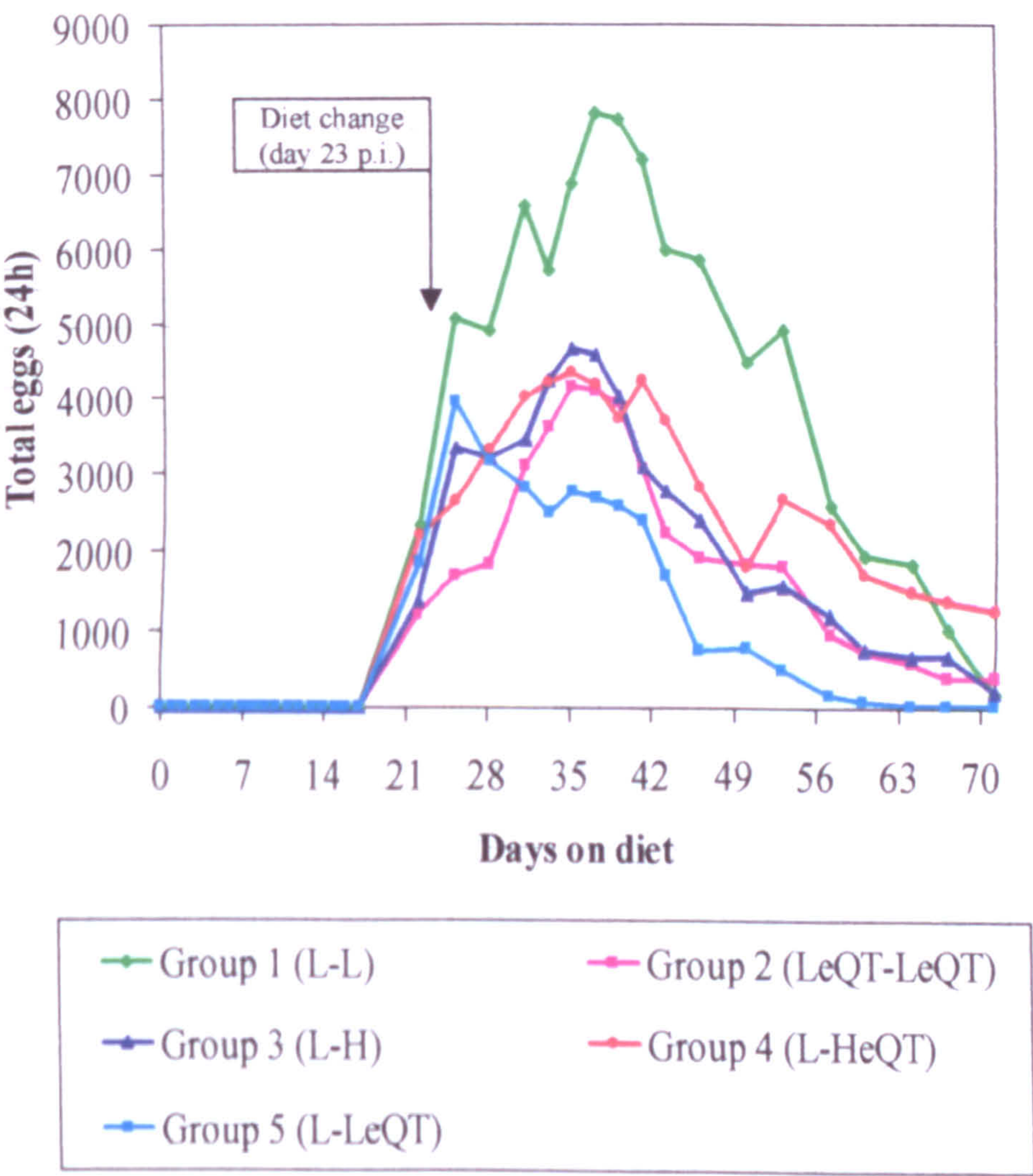
### 5.3.2 Faecal analysis

The faecal egg outputs of the infected groups are shown in Figure 5.2. Eggs were first seen on day 22 p.i. The following day was taken as the time point for diet change in groups 3, 4 & 5. Egg counts continued to increase up to a peak around day 35 p.i. and declined thereafter. Lambs in group 1 which were maintained on the diet L throughout the study had a significantly higher mean number of eggs per gram of dry faeces (EPG) after diet change (day 23-71 p.i., Table 5.4) than other treatment groups. The inclusion of eQT in diet L from day 23 p.i. onwards (group 5) resulted in these lambs passing the fewest nematode eggs, primarily due to a lower peak egg output. The addition of eQT to the diet prior to nematode infection (group 2) did not further reduce egg output. Increasing the protein content of the diet from 97 to 222 g/kg significantly reduced ( $p<0.001$ ) egg output compared to the low protein fed animals (group 1). However, including tannin in diet H (group 4) did not result in any further reduction in the EPG (Table 5.4). The uninfected controls (group 6) remained parasite-free throughout the experiment.

All infected lambs showed an erratic pattern of excretion of soft faeces from week 4 p.i. and egg output was therefore calculated on a dry faecal basis. Total daily faecal output shown in Table 5.4 was estimated from the chromium concentration in the same faecal grab samples collected for egg counting. Faecal output varied between animals and was highly dependent on the level of inappetence observed in the days prior to faecal collection, and also on the diet fed. Total faecal output using dungbags indicated that the average feed intake of the two days prior to the collection of faecal material produced faecal output estimates from chromium recovery that most closely reflected those from total collection. The inclusion of tannin in diet H (group 4) significantly increased ( $p<0.001$ ) faecal dry matter output, eQT did not increase faecal dry matter output in the low protein-fed lambs. Table 5.4 shows the estimated total daily egg outputs. The total daily nematode output was reduced by  $>50\%$  ( $p<0.01$ ) by eQT inclusion, both when included throughout (group 2) or introduced



**Figure 5.2** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or the inclusion of dietary quebracho tannin) on the total faecal egg output from lambs until anthelmintic treatment (day 75 p.i.)





**Table 5. 4** Effect of dietary treatment (high and low dietary protein concentration and/or dietary quebracho tannin) on mean faecal output and number of nematode eggs passed post diet change

| Group                                     | 1<br>(L-L)         | 2<br>(LeQT-LeQT)    | 3<br>(L-H)          | 4<br>(L-HeQT)       | 5<br>(L-LeQT)     | s.e.d.<br>(25 d.f.) |
|---|--------------------|---------------------|---------------------|---------------------|-------------------|---------------------|
| Eggs per gram<br>(faecal DM)              | 4716 <sup>a</sup>  | 2077 <sup>b,c</sup> | 2454 <sup>b,c</sup> | 2904 <sup>b,c</sup> | 1575 <sup>b</sup> | 589.2               |
| Faecal Output<br>(g DM/d)                 | 456 <sup>a</sup>   | 420 <sup>a</sup>    | 429 <sup>a</sup>    | 677 <sup>b</sup>    | 474 <sup>a</sup>  | 54.3                |
| Daily egg output<br>(x10 <sup>4</sup> DM) | 212.3 <sup>a</sup> | 104.8 <sup>b</sup>  | 112.2 <sup>b</sup>  | 204.0 <sup>a</sup>  | 83.3 <sup>b</sup> | 39.52               |

<sup>a,b,c</sup> means with different superscripts within a row are significantly different (p<0.05)

Values represent the average of 17 time points taken from day 23-71 p.i. for 6 animals in each infected group (1-5).



after worm establishment (group 5). Total daily egg output was decreased (47 %,  $p<0.05$ ) by increasing the protein content of the diet (group 3). Interestingly the inclusion of tannin in the high protein diet (group 4) had no effect ( $p>0.2$ ) on total egg output.

### 5.3.3 Blood analysis

#### 5.3.3.1 *Eosinophilia*

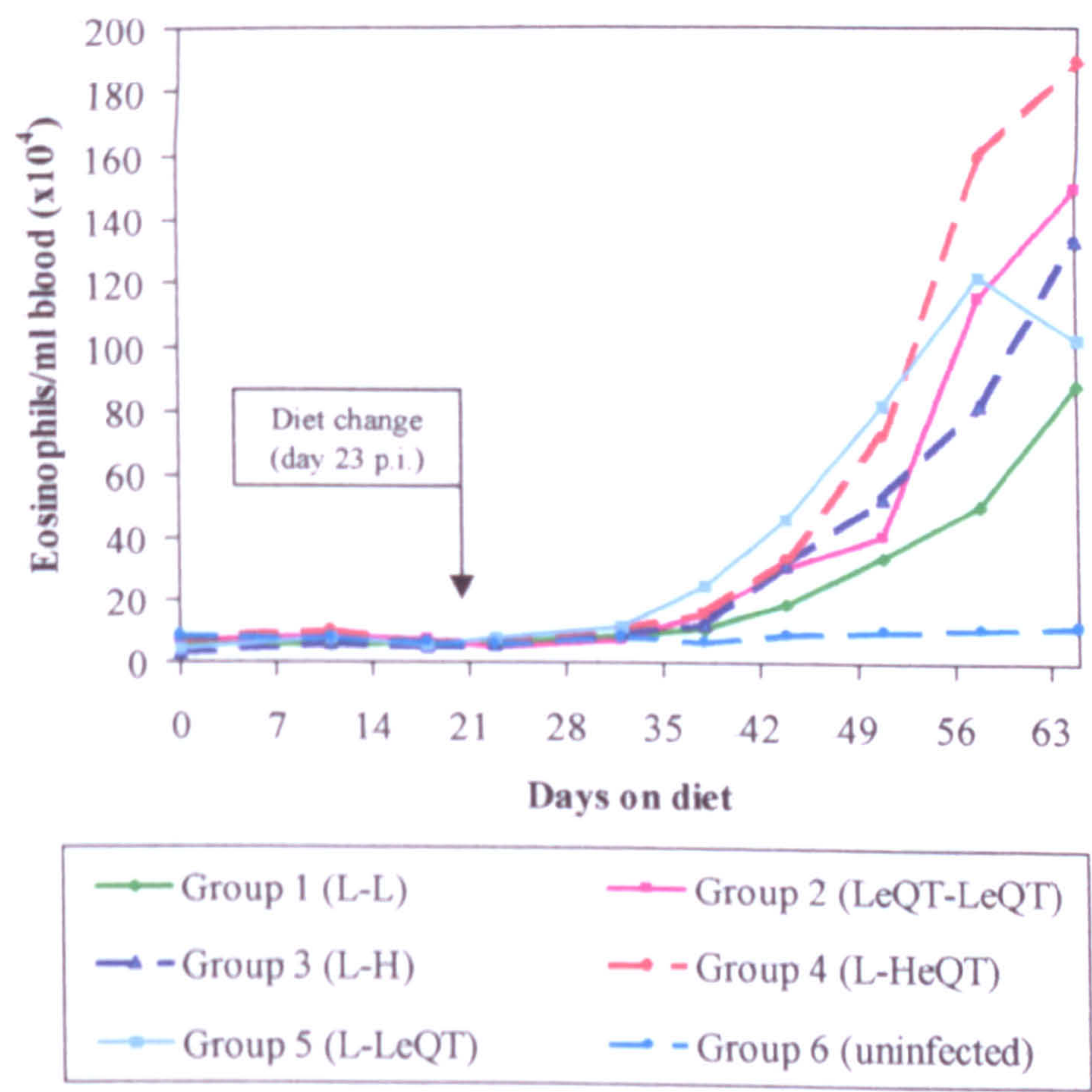
Peripheral eosinophil values remained low in all groups until day 38 p.i. and then increased with time in infected animals (groups 1-5, Figure 5.3). Eosinophilia in uninfected controls (group 6) remained low throughout. Lambs fed diet HeQT (group 4) showed the greatest response. The presence of eQT in both diet H and diet L tended to elevate the number of eosinophils circulating in peripheral blood of infected animals (Table 5.5). Of infected animals, lambs fed diet L throughout (group 1) had the lowest eosinophil concentration at all times.

#### 5.3.3.2 *Total protein, albumin and globulin*

Mean concentrations of total protein, albumin and globulin were determined for each animal between 23-71 days p.i. (Table 5.5). The profiles of total protein, albumin and globulin during the experimental period are shown in Figure 5.4. An increase in total protein concentration occurred around day 18 p.i. when the worms were establishing in the gut, the concentration then declined again before increasing from day 33 p.i. onwards (as faecal egg counts began to decline). Mean total protein concentration tended to be elevated in the infected animals (groups 1-5). Levels of albumin present in plasma tended to decrease in the infected groups (1-5) from day 38 p.i. onwards. Mean albumin concentrations of the uninfected animals (group 6) were significantly higher than infected lambs fed diet L  $\pm$  eQT (group 1, 2 & 5,  $p<0.05$ ). Diet H  $\pm$  eQT fed lambs (groups 3 & 4) tended to have a higher albumin concentrations than the infected low protein  $\pm$  eQT fed lambs, giving concentrations that were significantly similar to uninfected controls (group 6). Globulin



**Figure 5.3** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or the inclusion of dietary quebracho tannin) on the number of eosinophils circulating in peripheral blood of lambs until anthelmintic treatment (day 75 p.i.)





**Table 5. 5** Effect of *T. colubriformis* infection and dietary treatment (increasing dietary protein concentration and/or dietary quebracho tannin) on mean plasma profiles post diet change

| Group                                 | 1<br>(L-L)          | 2<br>(LeQT-LeQT)    | 3<br>(L-H)            | 4<br>(L-HeQT)       | 5<br>(L-LeQT)       | 6<br>(uninfected) | s.e.d.<br>(30 d.f) |
|---------------------------------------|---------------------|---------------------|-----------------------|---------------------|---------------------|-------------------|--------------------|
| Total protein (g/l)                   | 6.33 <sup>a,b</sup> | 6.56 <sup>a,b</sup> | 6.51 <sup>a,b</sup>   | 6.49 <sup>a,b</sup> | 6.60 <sup>a</sup>   | 6.11 <sup>b</sup> | 0.227              |
| Albumin (g/l)                         | 2.53 <sup>a</sup>   | 2.59 <sup>a</sup>   | 2.65 <sup>a,b</sup>   | 2.67 <sup>a,b</sup> | 2.59 <sup>a</sup>   | 2.85 <sup>b</sup> | 0.106              |
| Globulin (g/l)                        | 3.80 <sup>a</sup>   | 3.98 <sup>a</sup>   | 3.86 <sup>a</sup>     | 3.82 <sup>a</sup>   | 4.04 <sup>a</sup>   | 3.26 <sup>b</sup> | 0.212              |
| Eosinophils<br>(x10 <sup>4</sup> /ml) | 30.9 <sup>a,b</sup> | 52.0 <sup>b,c</sup> | 46.5 <sup>a,b,c</sup> | 69.7 <sup>c</sup>   | 56.6 <sup>b,c</sup> | 9.0 <sup>a</sup>  | 18.58              |

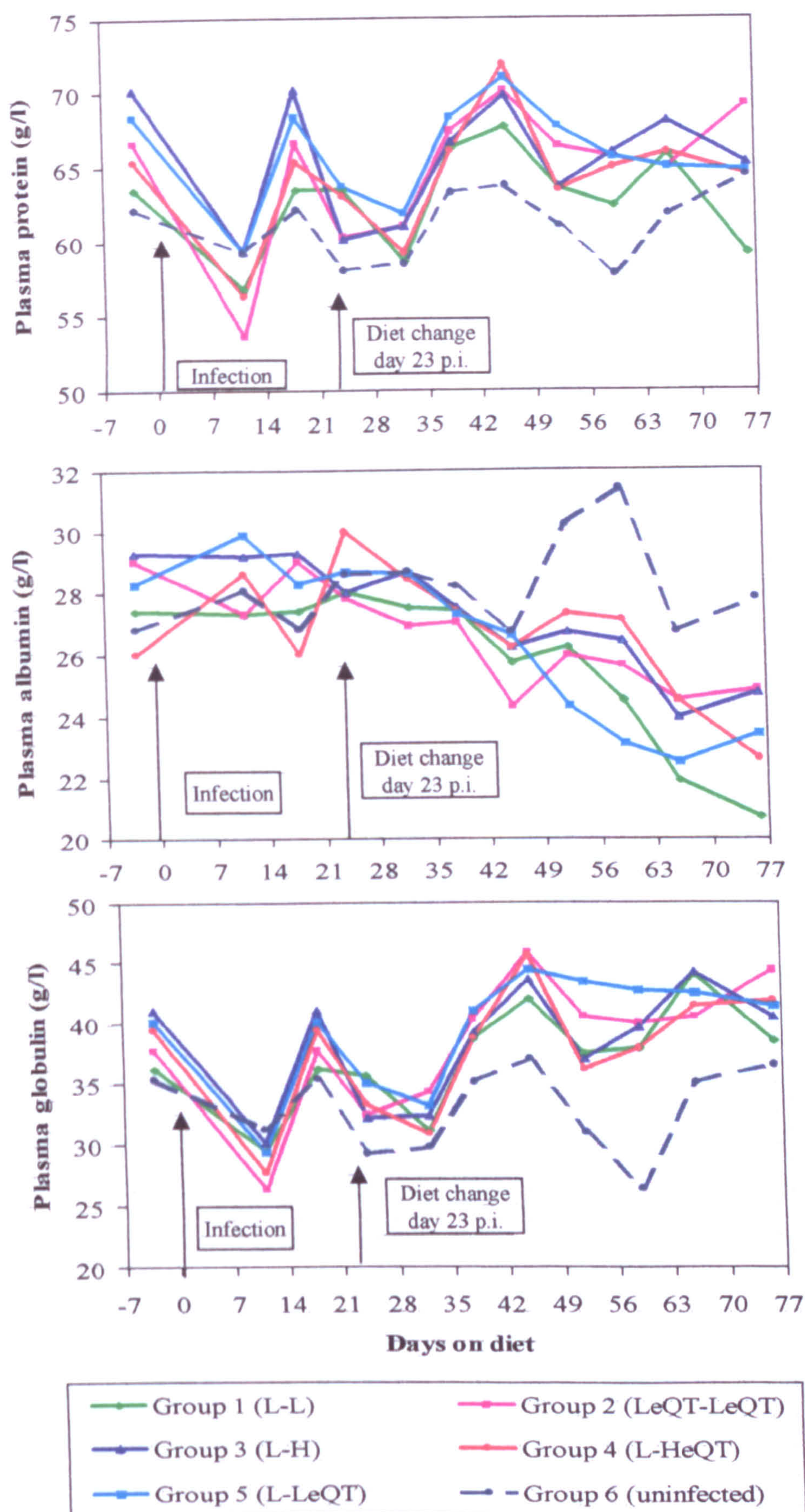
<sup>a,b,c</sup> means with different superscripts within a row are significantly different (p<0.05)

Values presented for total protein, albumin and globulin represent the average of 9 time points taken from day 23-71 p.i., values presented for eosinophils represent the average of 7 time points taken from day 23-71 p.i. for 6 animals in each group.

**Figure 5.4** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or dietary quebracho tannin) on total protein, albumin and globulin fractions in plasma from lambs.

Observations taken pre-infection until anthelmintic drenching (day 75 p.i.)







concentrations in the plasma tended to increase in parallel with the values observed for total protein, the increase in plasma globulin concentrations between infected animals (groups 1-5) compared to uninfected controls (group 6) being most distinct following peak egg production ( $p < 0.01$ ). No difference was seen between the infected groups.

#### *5.3.3.3 IgG response to ovalbumin*

All groups made IgG responses to ovalbumin, but overall there was no significant difference between the infected and control lambs (Table 5.6). Infected lambs fed diet HeQT (group 4) produced less specific IgG than any other group. The highest values were seen in the lambs fed diet L  $\pm$  eQT diets (group 1, 2 & 5).

#### **5.3.4 Slaughter**

At slaughter the gastrointestinal tract was removed and the small intestine processed for worm recovery. The empty warm carcass was then weighed and all carcass parameters measured expressed as a proportion of the recorded warm carcass weight (Table 5.7). Uninfected lambs (group 6) had the heaviest carcasses. Those lambs fed diet H  $\pm$  eQT (groups 3 & 4) tended to have lighter carcasses, but statistically were similar ( $p > 0.1$ ) to uninfected controls (group 6). The carcass weight of infected lambs fed diet L  $\pm$  eQT (groups 1, 2 & 5) were lighter than the carcasses of the uninfected controls (group 6,  $p < 0.05$ ). The killing out percentage was significantly depressed by infection ( $p < 0.05$ ), except for those lambs fed diet H (group 3). The inclusion of eQT in diet H (group 4) significantly reduced the killing out percentage compared to diet H-fed lambs (group 3,  $p < 0.05$ ). No significant differences ( $p > 0.1$ ) were seen for fat depot weights, with the exception of H + eQT fed lambs (group 4) having a smaller quantity of perirenal fat when expressed on a carcass weight basis compared to uninfected controls (group 6,  $p < 0.05$ ).



**Table 5. 6** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or dietary quebracho tannin) on mean IgG response to ovalbumin (see section 5.3.4.4)

| Group                       | 1                 | 2                 | 3                   | 4                 | 5                   | 6                   | s.e.d.   |
|-----------------------------|-------------------|-------------------|---------------------|-------------------|---------------------|---------------------|----------|
|                             | (L-L)             | (LeQT-LeQT)       | (L-H)               | (L-HeQT)          | (L-LeQT)            | (uninfected)        | (30 d.f) |
| OD <sup>1</sup><br>(410 nm) | 3.49 <sup>a</sup> | 3.33 <sup>a</sup> | 2.66 <sup>a,b</sup> | 1.84 <sup>b</sup> | 2.93 <sup>a,b</sup> | 2.59 <sup>a,b</sup> | 0.607    |

<sup>a,b</sup> means with different superscripts within a row are significantly different (p<0.05)

OD<sup>1</sup> Optical density scaled to a positive control having an optical density of 1.00

**Table 5.7** Effect of *T. colubriformis* infection and dietary change (increasing dietary protein concentration and/or dietary quebracho tannin) on carcass characteristics and worm burdens recovered from the small intestine at slaughter 10 days after the challenge infection

| Group                | 1                    | 2                    | 3                    | 4                      | 5                    | 6                         | s.e.d.   |
|----------------------|----------------------|----------------------|----------------------|------------------------|----------------------|---------------------------|----------|
|                      | (L-L)                | (LeQT-LeQT)          | (L-H)                | (L-HeQT)               | (L-LeQT)             | (uninfected) <sup>1</sup> | (30 d.f) |
| Carcass weight (kg)  | 28.12 <sup>a,b</sup> | 27.18 <sup>a</sup>   | 32.65 <sup>b,c</sup> | 29.59 <sup>a,b,c</sup> | 27.67 <sup>a,b</sup> | 34.65 <sup>c</sup>        | 2.502    |
| Killing out %        | 55.44 <sup>a,b</sup> | 55.60 <sup>a,b</sup> | 56.43 <sup>b,c</sup> | 53.47 <sup>a</sup>     | 54.70 <sup>a,b</sup> | 58.52 <sup>c</sup>        | 1.354    |
| Omental fat (g/kg)   | 50.0                 | 46.9                 | 52.7                 | 50.6                   | 46.9                 | 48.1                      | 6.89     |
| Perirenal fat (g/kg) | 34.5 <sup>a,b</sup>  | 33.0 <sup>a,b</sup>  | 38.1 <sup>a,b</sup>  | 28.7 <sup>a</sup>      | 33.4 <sup>a,b</sup>  | 39.7 <sup>b</sup>         | 5.05     |
| Worm burden          | 0                    | 10                   | 0                    | 207                    | 23                   | 16287 <sup>a</sup>        | 574.8    |

<sup>a,b</sup> means with different superscripts within a row are significantly different (p<0.05)

All lambs (groups 1-6) were drenched with anthelmintic of day 75 p.i. The lambs were all fed a low protein diet until slaughtered 10 days following the challenge infection (groups 1-6)

Group 6 (uninfected)<sup>1</sup> lambs remained uninfected during the trickle infection. Following anthelmintic treatment, group 6 lambs were also infected with the challenge dose



Subsamples of the number of *T. colubriformis* worms recovered from the small intestine of each lamb were counted and then multiplied to give the total worm burden. All those lambs which had been exposed to the trickle infection (groups 1-5) were resistant to the subsequent challenge infection (worm burdens very low). Those lambs, which remained uninfected during the trickle infection period, were susceptible to the challenge infection, with large numbers of *T. colubriformis* worms being recovered from the small intestine.

## 5.4 PRELIMINARY DISCUSSION

The aim of the second sheep study was to investigate the effects of increasing dietary protein concentration and/or the inclusion of dietary eQT on the ability of lambs to expel a primary infection and withstand a challenge infection of *T. colubriformis*. The general immune status of the lambs was measured through haematological and serological parameters.

The results of this study support previous observations that subclinical infections of *T. colubriformis* in sheep reduce performance, especially those maintained on low planes of nutrition (Poppi *et al.*, 1990; Kyriazakis *et al.*, 1994; van Houtert *et al.*, 1995b). In the present study infected animals fed diet L (97 g/kg) throughout finished the trial some 11 kg lighter than uninfected control group fed the same diet. This was at least partly due to a reduction in feed intake in infected lambs that occurred following worm establishment in the small intestine. The increase in dietary protein concentration from diet L (97 gCP/kg) to diet H (222 gCP/kg) compensated for the fall in production due to internal parasitism in the present study. Lambs abruptly changed to diet H  $\pm$  eQT (groups 3 & 4, day 23 p.i.) maintained liveweight gain similar to those animals in group 6 which remained uninfected and fed diet L.

Increasing the dietary protein concentration and hence the intestinal protein supply to infected animals has been shown to reduce liveweight losses and to enhance the host's resistance to further parasite infection (Wagland *et al.*, 1984; Bown *et al.*, 1991b; Abbott *et al.*, 1986, 1988; Kambara *et al.*, 1993), and data from the present study support these observations. Reduced nematode burdens have also been reported in sheep grazing forages containing condensed tannins (Niezen *et al.*, 1995; Robertson *et al.*, 1995). This has usually been attributed to an improved protein supply in tannin-fed animals, although it has also been suggested that the condensed tannin may be acting directly against the nematodes (Niezen *et al.*, 1993, 1995). The data from the present study show that both increased dietary protein concentration and dietary tannin inclusion reduced nematode burden but the effects were not



additive. Indeed, in lambs fed diet HeQT (group 4) daily faecal egg output was similar to control animals fed diet L (group 1) suggesting that in combination the beneficial effects of tannin and protein were neutralised. One group of lambs received eQT before and after infection to determine whether eQT affected worm establishment (group 2). Compared to animals fed eQT after establishment (group 5), those animals that received tannin throughout had a depressed faecal egg count up to day 29 p.i. but no further benefit seen thereafter suggesting that the tannin had only a slight effect on worm establishment.

Although the plasma profiles of total protein, albumin and globulin concentrations differed between infected and uninfected groups, no dietary interactions were seen. The fact that plasma profiles differed only in relation to parasite infection suggests that dietary tannin does not significantly increase endogenous protein secretions by the gastrointestinal tract, unless increased endogenous secretions are compensated by increased protein availability due to protected passage through the rumen.

Infection with *T. colubriformis* was associated with a marked elevation in peripheral eosinophilia, an important indicator of helminth infection associated with the expulsion of the parasite from the gut (Dawkins *et al.*, 1989). Eosinophilia was only seen once a decline in faecal egg counts had occurred and there was no significant correlation with dietary protein content, in agreement with Kyriazakis *et al.* (1996b). However, there was a tendency for animals fed diet H (groups 3 & 4) to have elevated eosinophilia whether or not eQT was present. The inclusion of eQT in the diet tended to elevate eosinophil concentrations, this being more apparent than the effect of protein in the diet. The combination of feeding diet HeQT (group 4) had an additive effect, resulting in a statistically significantly high eosinophilia compared to uninfected controls (group 6) and infected lambs fed diet L throughout (group 1). Although, increased numbers of tissue and circulating eosinophils during helminth infection have been suggested to provide an index of protective immune response to parasitism (Datta *et al.*, 1998), those lambs fed diet LeQT (groups 2 & 5) had only a slight elevation in circulating eosinophil concentrations but a 50% decrease in total daily nematode egg output compared to infected animals fed diet L throughout

(group 1). This could indicate that the inclusion of dietary eQT may be acting locally on the intestinal epithelium increasing the numbers of tissue eosinophils which precede an elevation in peripheral eosinophil levels (Winter *et al.*, 1997). Tissue eosinophilia, as blood eosinophilia, is associated with the responsiveness of sheep infected with *T. colubriformis* (Rothwell *et al.*, 1993), and has been implicated in the rejection of worms from the gut in secondary infection (Stevenson *et al.*, 1994; Jones, 1993). However, this is not supported by those lambs fed diet HeQT (group 4) which had a significant elevation in eosinophil concentrations but no decline in daily nematode egg output compared to lambs fed diet L throughout (group 1).

Neither infection, protein concentration nor dietary eQT significantly influenced the level of circulating antibodies to ovalbumin, although, in both diet H and L, the inclusion of eQT tended to decrease the responsiveness of the animal to ovalbumin. Thus, it would appear that the inclusion of dietary eQT does not elevate the general immune responsiveness of the animals, making it unlikely that eQT reduces *T. colubriformis* infection in lambs via a direct effect on the immune system.

The results show that the carcass weight of the lambs mirrored those of liveweight, with uninfected controls being the heaviest. Feeding diet H to infected animals increased carcass weight to a value similar to uninfected diet L-fed lambs. Dietary treatment had no influence on fat depot measurements. The literature reports contrasting results on the effect of dietary condensed tannins on carcass fatness of ruminants. Purchas & Keogh (1984) found that sheep grazing *Lotus pedunculatus* (10-30g condensed tannin/kg DM) had reduced carcass fatness, while Terrill *et al.* (1992a) and Douglas *et al.* (1995) saw no response in carcass fatness in sheep grazing sulla (40-50 g condensed tannin/kg DM) and *Lotus corniculatus* (~40 g condensed tannin/kg DM) respectively, deer also showed no response when grazing *L. corniculatus* (48 g condensed tannin/kg organic matter; Min *et al.*, 1997). The worm burden recovered following challenge infection showed that those lambs exposed to the trickle infection had acquired a suitably large immune response against *T. colubriformis* infection to prevent a challenge infection from establishing in the small intestine.



In summary, the results from this chapter indicate that the elevation in dietary protein concentration to parasitised animals enables the animal to overcome some of the deleterious effects of infection. The inclusion of dietary eQT results in a similar reduction in faecal egg counts as seen from lambs fed diet H compared to infected animals fed diet L. The addition of eQT to diet H was not additive and returned total daily faecal egg counts to levels observed from infected L-fed lambs. The haematological parameters measured during the study did not show any elevation in immune responsiveness of animals fed eQT diets indicating that dietary eQT does not promote the responsiveness of the host immune system. Further explanations as to possible mechanism through which eQT is reducing nematode burden is discussed in the following chapter.

# **CHAPTER 6**

## **STUDIES TO INVESTIGATE THE MECHANISM OF ACTION OF QUEBRACHO TANNIN IN REDUCING NEMATODE BURDEN**

### **(RAT TRIALS 4 & 5 AND *IN VITRO* EXPERIMENTS)**

#### **6.1 INTRODUCTION**

The previous chapters have shown that including quebracho tannin in the diet can reduce the intensity of small intestinal infection with *Nippostrongylus brasiliensis* in the rat and *Trichostrongylus colubriformis* in the ovine host. While it is apparent that dietary quebracho tannin is exerting a negative effect on worm infection the mechanism through which this occurs remains unknown. Studies published by New Zealand workers (Robertson *et al.*, 1995; Niezen *et al.*, 1995) concluded that sheep naturally infected with gastrointestinal nematodes benefited from grazing forages containing condensed tannins. This was suggested to have been through condensed tannins protecting dietary protein from rumen and increasing post-ruminal protein supply (Niezen *et al.*, 1995). The possibility of condensed tannins or some other plant component having a direct effect against the worm burden was also suggested by Niezen *et al.* (1994). The reduction in *N. brasiliensis* infection in tannin-fed rats in chapter 3 suggest that dietary quebracho tannin is likely to be acting through a mechanism other than the possible elevation in protein supply to the small intestine of the ruminant host. Two possible alternatives are through the promotion of the host immune response or through a direct toxic effect against the infection.

Results published by Dawson *et al.* (1999) showed that quebracho tannin was taken up by the Peyer's patches of the small intestine. Antigens enter Peyer's patches through specialised cells and stimulate antigen-specific lymphocytes. One



consequence of their activation is that IgA is secreted into the gut lumen (Weir & Stewart, 1993), hence it was postulated that the uptake of quebracho tannin into Peyer's patches could induce a local immune response. Activation of the immune system may improve the ability of the animal to withstand disease including parasitic infection. To demonstrate whether dietary quebracho tannin is reducing nematode infections through the up-regulation of the immune system a further rat trial was carried out. Groups of tannin and control-fed rats were infected with *N. brasiliensis*. Around the time of infection the immune system of half the rats fed each diet was suppressed. If dietary quebracho tannin acted on infection by up-regulating the immune response, faecal egg counts from tannin-fed immune-suppressed rats should be similar to those of control-fed rats. Eosinophilia data from both sheep studies (Chapters 4 & 5) and ELISA data reported in chapter 5 tend to imply that dietary quebracho tannin had no effect on the host immune system.

Alternatively, quebracho tannin could have direct toxic effects against the nematode infection. This was determined by *in vivo* and *in vitro* experiments. Rats were infected either with *N. brasiliensis*, a lumen dwelling worm, or *Trichinella spiralis*, a mucosal inhabiting worm. If quebracho tannin is acting through a direct toxicity effect then *T. spiralis*, being only exposed to tannin for a brief period of time prior to burrowing into the gut mucosa, is less likely to be effected by the presence of dietary quebracho tannin in the digesta.

*In vitro* experiments monitoring the survival of adult *N. brasiliensis* incubated in varying concentrations of quebracho tannin, both eQT and uQT, were carried out providing further information of any direct toxicity effect of quebracho tannin against the worm.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Trial 4 investigating the effect of dietary quebracho tannin in the immune-suppressed rat

#### 6.2.1.1 *Animals and diet*

Twenty-eight male Wistar rats (initial weight  $114.66 \pm 4.64$  g) were given 5 days to acclimatise to their new environment before the introduction of diet L (low protein; Table 6.1). Seven days later the rats were randomly allocated to one of four experimental groups each containing 7 animals. Two groups of rats were maintained on diet L, the remaining two groups were fed diet L containing 40g extracted quebracho tannin/kg (L+eQT) which replaced 40g alphacel/kg (see Table 6.1). The rats were individually housed and fed *ad libitum* as described in section 2.2.1.1. Bodyweights and feed intakes were recorded three times weekly [Ohaus GT 4800 electronic balance, New Jersey, USA,].

#### 6.2.1.2 *Nematode infection*

All animals were maintained on the experimental diets for 16 days after the introduction of eQT, before being subcutaneously injected with 2000 infective (L<sub>3</sub>) *N. brasiliensis* suspended in PBS (section 2.3.1.).

#### 6.2.1.3 *Experimental protocol*

One group of rats from each of the control and tannin dietary treatments was immune suppressed before and post infection (p.i.). Immune suppression was induced by subcutaneous injection with Solu-Cortef<sup>®</sup> (hydrocortisone sodium succinate [Pharmacia & Upjohn Ltd., Milton Keynes, UK]). Solu-Cortef<sup>®</sup> was diluted with sterile water to give a final concentration of 12.5 mg/ml. Each rat, in the two immune



**Table 6. 1** Composition of the synthetic diets fed to rats (g/kg)

| Ingredient             | Supplier                      | Low protein | Low protein + eQT |
|------------------------|-------------------------------|-------------|-------------------|
| Maize Starch           | Dickens, Nottingham.          | 419         | 419               |
| Casein                 | Bacarel, Essex.               | 100         | 100               |
| Glucose                | Dickens, Nottingham.          | 300         | 300               |
| Alphacel               | ICN, USA                      | 80          | 40                |
| Mins/Vits              | SDS, Witham, Essex.           | 50          | 50                |
| L-methionine           | Sigma, Dorset.                | 1           | 1                 |
| Corn Oil               | Sainsbury's                   | 50          | 50                |
| Quebracho Tannin (eQT) | Hodgesons Chemicals Ltd. Hull | 0           | 40                |

suppression groups, was subcutaneously injected with 0.2 ml (2.5 mg hydrocortisone sodium succinate) on days -1, 1, 3 and 5 p.i. The remaining two groups of rats, unsuppressed controls, were subcutaneously injected with 0.2 ml sterile water on these days.

Faecal egg counts (eggs per gram, EPG) were estimated for all rats between day 5 and 7 p.i. as described previously (section 2.3.2.1). Sixteen hours before the first faecal egg count all faeces in the faecal tray were removed. The following day (day 5 p.i.), faeces were collected from the faecal tray. The weight of faeces passed over the previous 16h was recorded and approximately 1 g faeces were retained for egg counting. The remaining faeces were discarded leaving the faecal tray empty. Total faecal collection and egg counting were repeated 16h later, and then again after a further 16h. On day 7 p.i. all rats were killed and the worm burden recovered (section 2.3.3.1.). Once the small intestine was being incubated for worm recovery, faecal pellets were removed from the large intestine for the final faecal egg count. Total faecal egg output was estimated for each 16 h period (EPG x total faecal output of the proceeding 16 h) with the exception of the final egg count where faecal material for egg counting was removed directly from the large intestine.

## **6.2.2 Trial 5 investigating the effect of dietary quebracho tannin on the number of lumen dwelling (*N. brasiliensis*) and mucosal inhabiting (*T. spiralis*) worms established in the small intestine of rats**

### **6.2.2.1 Animals and diet**

Forty-eight male Wistar rats (initial mean bodyweight  $128.87 \pm 10.18$  g) were given 7 days to acclimatise to their new environment before the introduction of the diet L (low protein; Table 6.1). Seven days later the rats were randomly allocated to one of six experimental groups each containing 8 animals. Three groups of rats were fed diet L containing 40g eQT/kg which replaced 40g alphacel/kg (L+eQT; see Table 6.1), the remaining three groups continued to be fed diet L. The rats were



individually housed and fed *ad libitum* as described in section 2.2.1.1. Bodyweights and feed intakes were recorded three times weekly [Ohaus GT 4800 electronic balance, New Jersey, USA,].

#### 6.2.2.2 Nematode infection

All animals were maintained on the experimental diets for 16 days after the introduction of eQT. Thirty-two animals, 16 rats fed the control diet and 16 rats fed the tannin diet were orally infected with approximately 1000 L<sub>3</sub> *T. spiralis* suspended in 0.2% agar and administered via gavage. The remaining 16 animals (8 control-fed rats and 8 tannin-fed rats) were subcutaneously injected with approximately 2500 L<sub>3</sub> *N. brasiliensis* suspended in PBS as described in section 2.3.1.

#### 6.2.2.3 Experimental protocol

All rats were killed and the worm burdens recovered as described in section 2.3.3.1. Sixteen *T. spiralis* infected rats (8 control-fed and 8 tannin-fed animals) were killed on day 2 p.i. The remaining 16 *T. spiralis* infected rats (8 control-fed and 8 tannin-fed animals) were killed on day 5 p.i. The *N. brasiliensis* infected rats (8 control-fed and 8 tannin-fed animals) were killed on day 5 p.i.

### 6.2.3 Statistical Analysis

Data from each trial were analysed as a completely randomised experiment using one-way analysis of variance comparing control and tannin-fed groups. Daily liveweight gains were calculated by linear regression for each animal, slopes were tested by one-way analysis of variance. Data were blocked for rat and differences were assumed to be significant at  $p < 0.05$ .

#### **6.2.4 The survival of adult *N. brasiliensis* worms *in vitro* incubated in varying concentrations of quebracho tannin**

##### ***6.2.4.1 Adult worm extraction***

Additional male Wistar rats ordered for trial 5 were held in stock and fed a standard rodent diet (Special Diet Services, Witham, Essex). These rats were used to provide adult *N. brasiliensis* worms for *in vitro* experiments. Six days before the adult worms were required one rat was subcutaneously injected with approximately 2000 *N. brasiliensis* L<sub>3</sub> suspended in PBS. The rat was killed on the morning of the study and the worms recovered from the small intestine (section 2.3.3.1)

##### ***6.2.4.2 Index of worm survival***

Known numbers of adult worms (between 10 and 20 worms of mixed sex) were incubated in petri dishes containing 2 ml Hanks' balanced salt solution (HBSS, see appendix) and varying concentrations of quebracho tannin (eQT or uQT) ranging from 0.0% tannin (control) through to 4.0% (w/v) tannin as described in section 2.3.5. Experiment 1 involved varying quebracho tannin concentrations of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% (w/v) with both eQT and uQT. Quebracho tannin concentrations (eQT & uQT) in experiment 2 ranged from 0 to 1% (0.00, 0.125, 0.25, 0.50, 0.75, 1.00% (w/v)). The third experiment used only eQT. Concentrations ranged from 0 % to 1 % eQT (0.00, 0.01, 0.05, 0.125, 0.25, 0.5 and 1.0% (w/v)). In the third experiment all cultures were prepared in triplicate. A second set of triplicate cultures, at the same concentrations of eQT, were prepared in parallel with HBSS containing 0.1% (w/v) polyethylene glycol (PEG, MW 3350).

The motility of the worms was assessed as described in section 2.3.5 at regular intervals over a time period of up to 10h. The time at which 50% of worms were classed as no longer motile was determined from a quadratic equation where the intercept was set at 100 (all worm motile).



## 6.3 RESULTS

### 6.3.1 Trial 4 investigating the effect of dietary quebracho tannin in the immune-suppressed rat

#### 6.3.1.1 Liveweight and feed intake

The bodyweight of rats during the trial was reduced by the inclusion of dietary eQT. The average liveweight gain and feed intake of the rats are shown in Table 6.2. A significant depression in liveweight gain was seen in those rats fed dietary eQT ( $p<0.001$ ), but feed consumption was not significantly affected by dietary eQT.

#### 6.3.1.2 Faecal egg counts

Immune-suppression of the rats did not alter mean total faecal egg counts (16h period) or worm burdens recoveries in either control or tannin-fed rats (Table 6.3). The inclusion of dietary eQT reduced faecal egg counts by approximately 50% when presented as eggs per gram faeces (EPG) and also as total number of eggs (16h period). The recovery of worms from the small intestine of eQT-fed rats was approximately 70% of those recovered from the control fed rats ( $p<0.01$ ).

### 6.3.2 Trial 5 investigating the effect of dietary quebracho tannin on the number of lumen dwelling (*N. brasiliensis*) and mucosal inhabiting (*T. spiralis*) worms established in the small intestine of rats

#### 6.3.2.1 Liveweight and feed intake

The average daily liveweight gain of the rats is shown in Table 6.4, dietary eQT inclusion in diet L reduced growth rate ( $p<0.01$ ). Quebracho tannin fed rats tended to consume less feed, only those rats infected with *T. spiralis* and killed 2 days p.i.

**Table 6. 2** Effect of dietary eQT on growth rate and feed intake of normal and immune-suppressed rats (Trial 4).

|                             | Growth rate (g/d) | Feed Intake (g/d) |
|-----------------------------|-------------------|-------------------|
| L                           | 4.43 <sup>a</sup> | 20.47             |
| L - immune suppressed       | 4.27 <sup>a</sup> | 20.31             |
| L + eQT                     | 3.59 <sup>b</sup> | 19.10             |
| L + eQT - immune suppressed | 3.56 <sup>b</sup> | 19.26             |
| s.e.d. (26 d.f.)            | 0.317             | 1.111             |

<sup>a,b</sup>means with different superscripts within a column are significantly different p<0.05.

Results are means of 9 observations from the introduction of dietary tannin into appropriate animal groups to 10 days p.i.



**Table 6. 3** Effect of dietary eQT on mean faecal egg counts and the number of adult *N. brasiliensis* worms recovered from the small intestine of normal and immune-suppressed rats (Trial 4).

|                             | EPG                | Total eggs (16 h)    | Worm Burden       |
|-----------------------------|--------------------|----------------------|-------------------|
| L                           | 24577 <sup>a</sup> | 52197 <sup>a</sup>   | 1089 <sup>a</sup> |
| L - immune suppressed       | 27928 <sup>a</sup> | 50841 <sup>a,b</sup> | 1133 <sup>a</sup> |
| L+eQT                       | 10120 <sup>b</sup> | 27434 <sup>b,c</sup> | 728 <sup>b</sup>  |
| L + eQT - immune suppressed | 9670 <sup>b</sup>  | 23341 <sup>c</sup>   | 739 <sup>b</sup>  |
| s.e.d. (24 d.f.)            | 5231.1             | 12698.1              | 118.6             |

<sup>a,b,c</sup>means with different superscripts within a column are significantly different p<0.05.

Eggs per gram faeces (EPG) are the mean of 4 observations.

Total eggs (16h) are the mean of 3 observations, no total faecal output established for the final EPG and therefore not included.

**Table 6. 4** Effect of dietary eQT on growth rate and feed intake of *T. spiralis* and *N. brasiliensis* infected rats (Trial 5).

|                                     | Growth rate (g/d)   |                     | Feed intake (g/d)  |                    |
|-------------------------------------|---------------------|---------------------|--------------------|--------------------|
|                                     | L                   | L + eQT             | L                  | L +eQT             |
| <i>T. spiralis</i> , day 2 p.i.     | 4.30 <sup>a</sup>   | 3.28 <sup>b,c</sup> | 20.15 <sup>b</sup> | 18.82 <sup>b</sup> |
| <i>T. spiralis</i> , day 5 p.i.     | 3.68 <sup>a,b</sup> | 2.90 <sup>c</sup>   | 19.89 <sup>b</sup> | 19.63 <sup>b</sup> |
| <i>N. brasiliensis</i> , day 5 p.i. | 3.75 <sup>a,b</sup> | 3.35 <sup>b,c</sup> | 20.89 <sup>a</sup> | 19.88 <sup>b</sup> |
| s.e.d. (46 d.f.)                    | 0.347               |                     | 0.835              |                    |

<sup>a,b,c</sup>means with different superscripts within a column are significantly different p<0.05.

Results are means of 10-11 observations from the introduction of dietary tannin in appropriate animal groups to 10 days p.i.



significantly consumed less feed ( $p < 0.05$ ) than the control-fed *N. brasiliensis* infected rats.

#### 6.3.2.1 Worm burden

The worm burdens recovered from the small intestine are presented in Table 6.5. For each experimental group there were worm burdens from rats which were beyond 2 standard deviations from the mean, presumably due to variability at infection. Consequently the data generated from these rats were removed from the analysis giving different degrees of freedom from that expected (11 or 14 d.f. rather than 16 d.f.). The infection of rats with *T. spiralis* resulted in over 90% of the infected dose being recovered 2 days after infection. By day 5 p.i. approximately 30% fewer worms were recovered from the small intestine compared to the burden recovered on day 2 p.i. At both time points the inclusion of dietary eQT had no influence ( $p > 0.1$ ) on the numbers of *T. spiralis* recovered from the small intestine. The *N. brasiliensis* worm burden recovered from L + eQT-fed rats however, was significantly lower ( $p < 0.001$ ) than that recovered from rats fed the control diet.

#### 6.3.3 The survival of adult *N. brasiliensis* worms *in vitro* incubated in varying concentrations of quebracho tannin

Experiment 1 (*N. brasiliensis* worms incubated in eQT and uQT between 0.0 and 4.0% (w/v)) showed that worms of both sexes remained active and viable when incubated in 2 ml HBSS for periods of up to 4h, after which some worms began to become inactive (no body movement and not responding to mechanical stimulation). The solubility of uQT was considerably less than eQT in HBSS. The solubility of eQT in HBSS was exceeded at 4% (w/v) but uQT reached maximum solubility at only 2% (w/v). Tannin precipitation from solution also increased with time. Consequently, worms incubated in cultures prepared beyond these concentrations were not visible. Results shown in Figures 6.1(A, B) & 6.2(A, B) show the rate of mortality of worms incubated in control media and varying concentrations of eQT or uQT for experiments 1 & 2. The activity of *N. brasiliensis* was compromised at all

**Table 6. 5** Effect of dietary eQT on the number of *T. spiralis* and *N. brasiliensis* worms recovered from the small intestine of rats (Trial 5)

|                                   | L                 | L + eQT           | s.e.d. (d.f.) |
|-----------------------------------|-------------------|-------------------|---------------|
| <i>T. spiralis</i> , d 2 p.i.     | 935               | 930               | 84.9 (14)     |
| <i>T. spiralis</i> , d 5 p.i.     | 675               | 614               | 90.9 (14)     |
| <i>N. brasiliensis</i> , d 5 p.i. | 1442 <sup>a</sup> | 1090 <sup>b</sup> | 82.8 (11)     |

<sup>a,b</sup>means with different superscripts within a row are significantly different p<0.05.

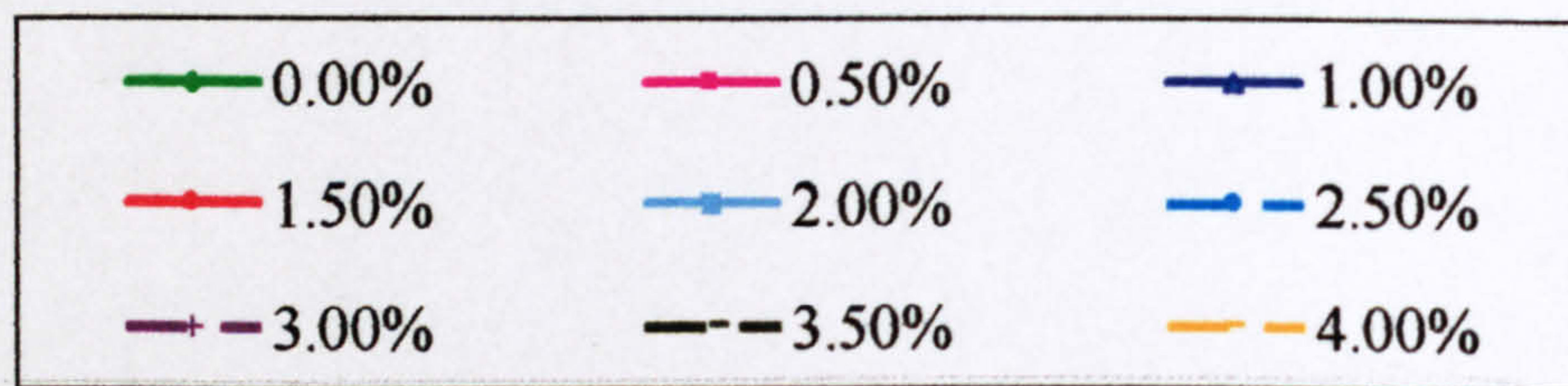


**Figure 6.1** Effect of varying concentrations of quebracho tannin in HBSS on the survival of adult *N. brasiliensis* worms *in vitro* (experiment 1)

**Graph A** Effect of increasing concentrations of eQT in HBSS on the survival of adult *N. brasiliensis* worms *in vitro*

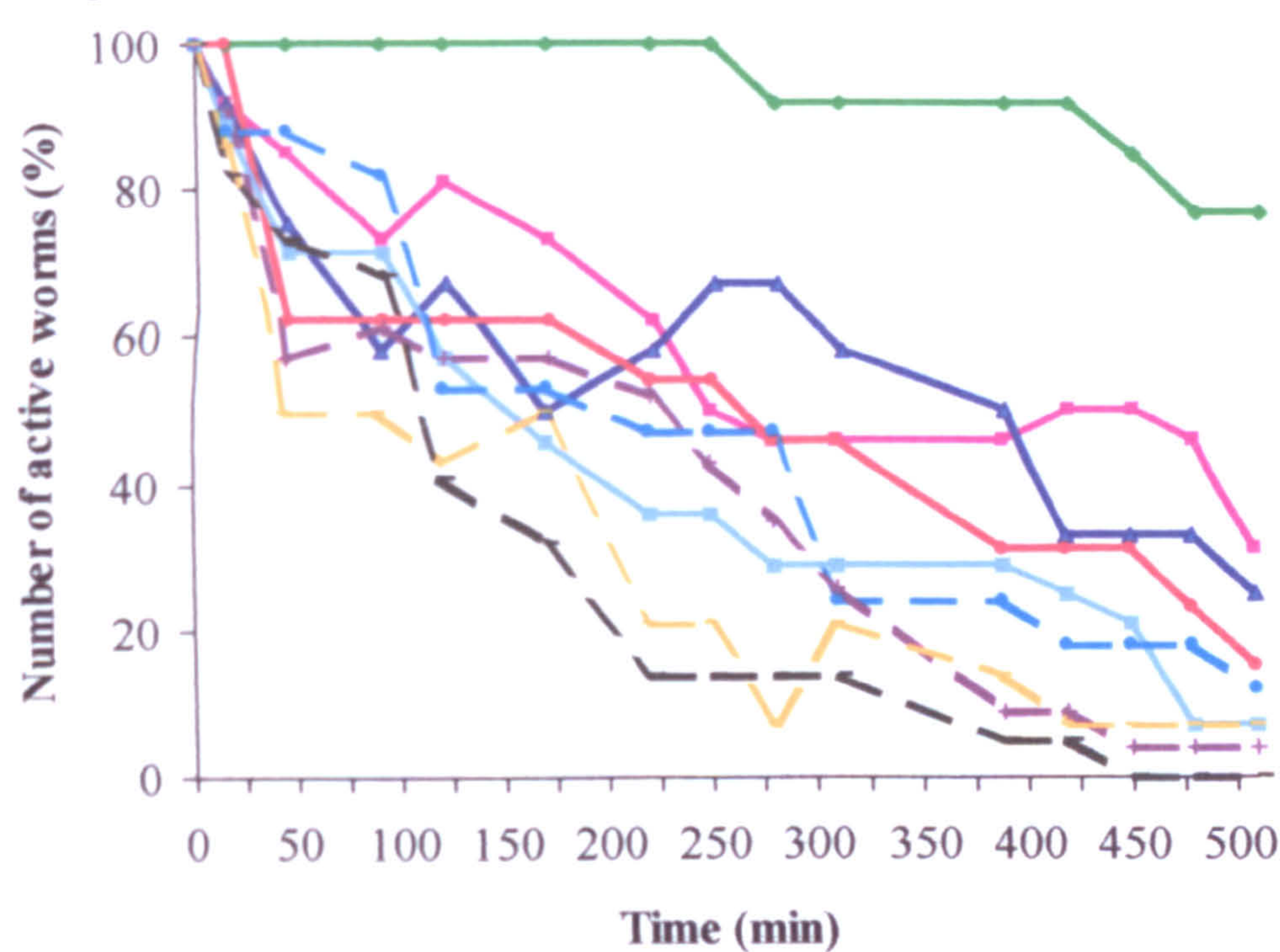
**Graph B** Effect of increasing concentrations of uQT in HBSS on the survival of adult *N. brasiliensis* worms *in vitro*

Legend for Graph 6.1 A & B

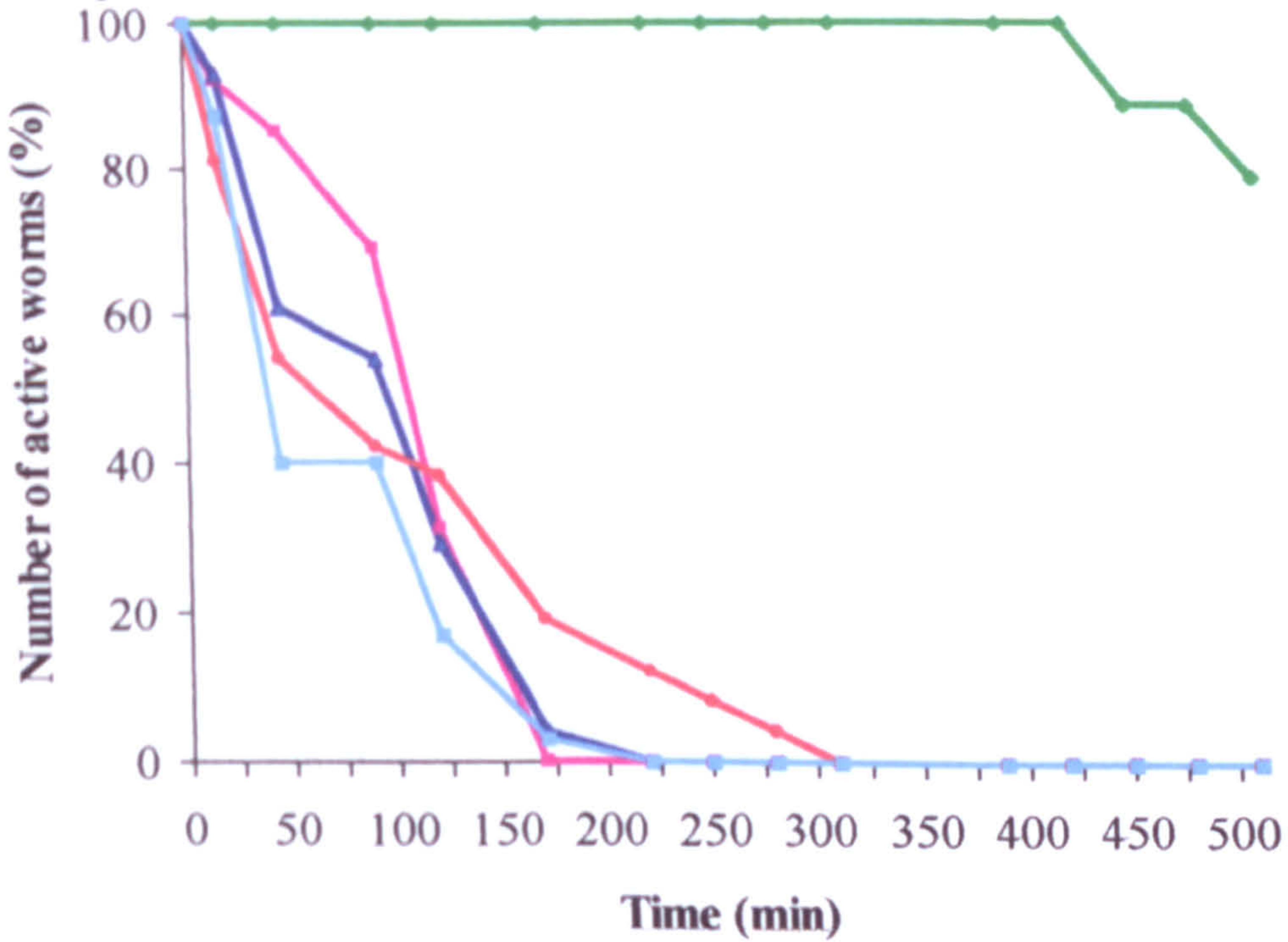




**Graph A**



**Graph B**





**Figure 6.2** Effect of varying concentrations of quebracho tannin in HBSS on the survival of adult *N. brasiliensis* worms *in vitro* (experiment 2)

**Graph A** Effect of increasing concentrations of eQT in HBSS on the survival of adult *N. brasiliensis* worms *in vitro*

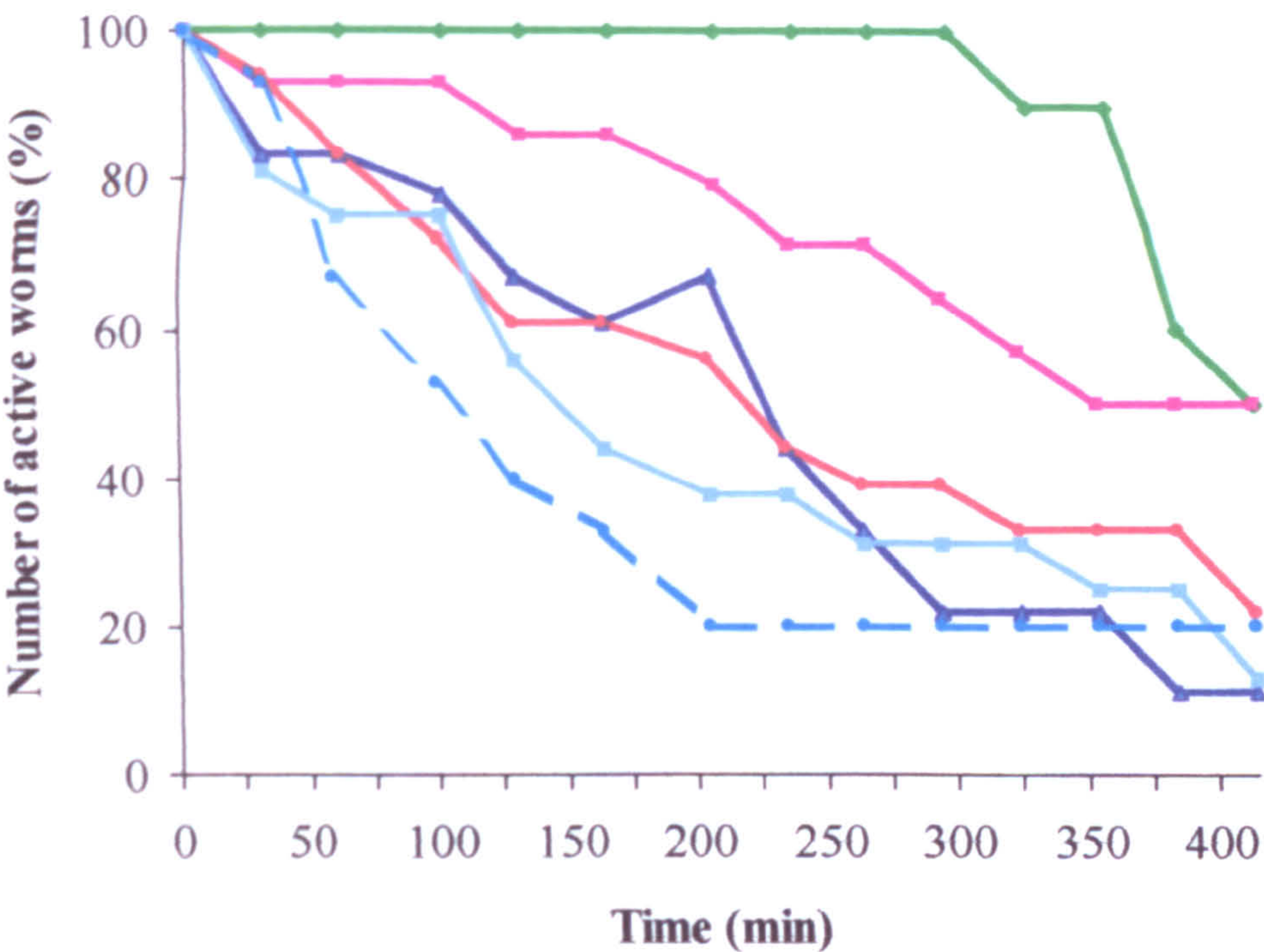
**Graph B** Effect of increasing concentrations of uQT in HBSS on the survival of adult *N. brasiliensis* worms *in vitro*

Legend for Graph 6.2 A & B

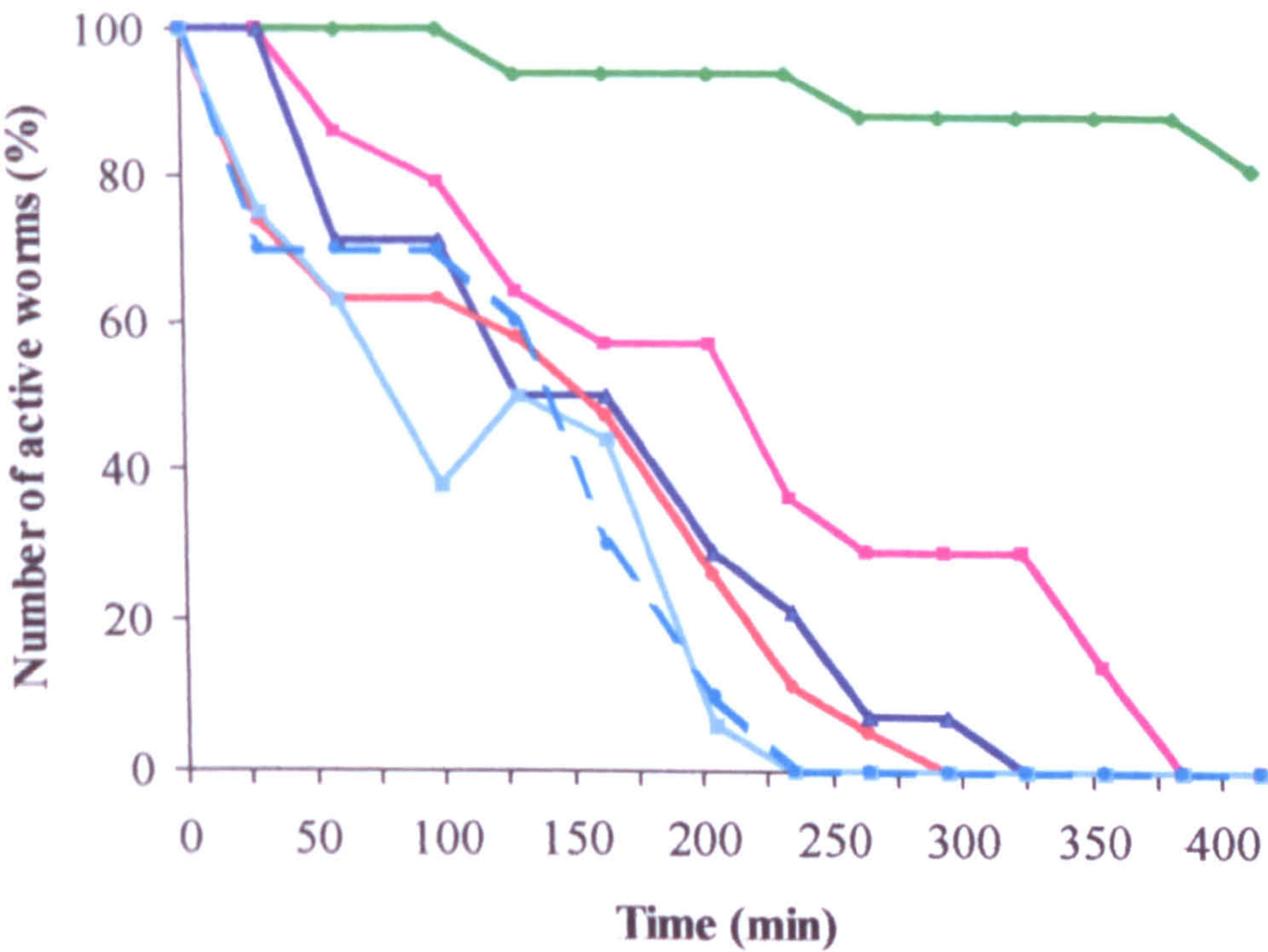




**Graph A**



**Graph B**





concentrations of quebracho tannin tested (eQT and uQT). Times for 50% of worms to die are shown in Table 6.6. Worms incubated in 0.5% (w/v) eQT reached 50% mortality in half the time period of worms incubated in HBSS (control culture) in both experiments 1 & 2. Increasing the concentration of eQT accelerated worm death. The presence of uQT in HBSS promoted worm death much more rapidly than eQT in both experiments 1 & 2. Worms incubated in 0.5% (w/v) uQT reached 50% mortality approximately four times faster than those incubated in 0.5% (w/v) eQT. Increasing concentrations of uQT above 0.125% (w/v) uQT did not greatly accelerate worm death further.

Experiment 3 compared worms incubated in increasing concentrations of eQT in the presence and absence of 0.1% (w/v) PEG. Concentrations as low as 0.01% (w/v) eQT tended to accelerate worm death (Figure 6.3 (A, B)). Time for 50% of *N. brasiliensis* worms to die are shown in Table 6.7. In the presence of PEG, 0.01% (w/v) eQT did not promote worm death. Concentrations of eQT greater than 0.01% (w/v) in the presence of PEG did reduce worm survival, although PEG did exert some protection over the possible toxic effect of eQT against *N. brasiliensis*.

During the experiments those worms incubated in quebracho tannin-containing media tended to display increased activity until death compared to those worms incubated in control media or very low concentrations of quebracho tannin (up to 0.1% (w/v)). In addition, worms incubated in quebracho tannin-containing media, generally had darkly-stained guts, presumably due to the intake of tannin into the digestive tract.

**Table 6. 6** Time for 50% of adult *N. brasiliensis* worms incubated in varying concentrations of quebracho tannin (eQT and uQT) in HBSS to die.

| eQT concentration<br>(%, w/v) | 50% mortality<br>(min) | uQT concentration<br>(%, w/v) | 50% mortality<br>(min) |
|-------------------------------|------------------------|-------------------------------|------------------------|
| Experiment 1                  |                        |                               |                        |
| 0.0                           | 714                    | 0.0                           | 804                    |
| 0.5                           | 314                    | 0.5                           | 98                     |
| 1.0                           | 294                    | 1.0                           | 94                     |
| 1.5                           | 235                    | 1.5                           | 102                    |
| 2.0                           | 176                    | 2.0                           | 88                     |
| Experiment 2                  |                        |                               |                        |
| 0.00                          | 418                    | 0.00                          | 354                    |
| 0.125                         | 384                    | 0.125                         | 83                     |
| 0.25                          | 207                    | 0.25                          | 55                     |
| 0.50                          | 219                    | 0.50                          | 49                     |
| 0.75                          | 170                    | 0.75                          | 41                     |
| 1.00                          | 118                    | 1.00                          | 44                     |



**Figure 6.3** Effect of varying concentrations of eQT in HBSS with and without 0.1% (w/v) PEG on the survival of adult *N. brasiliensis* worms *in vitro* (experiment 3)

**Graph A** Effect of increasing concentrations of eQT in HBSS on the survival of adult *N. brasiliensis* worms *in vitro*

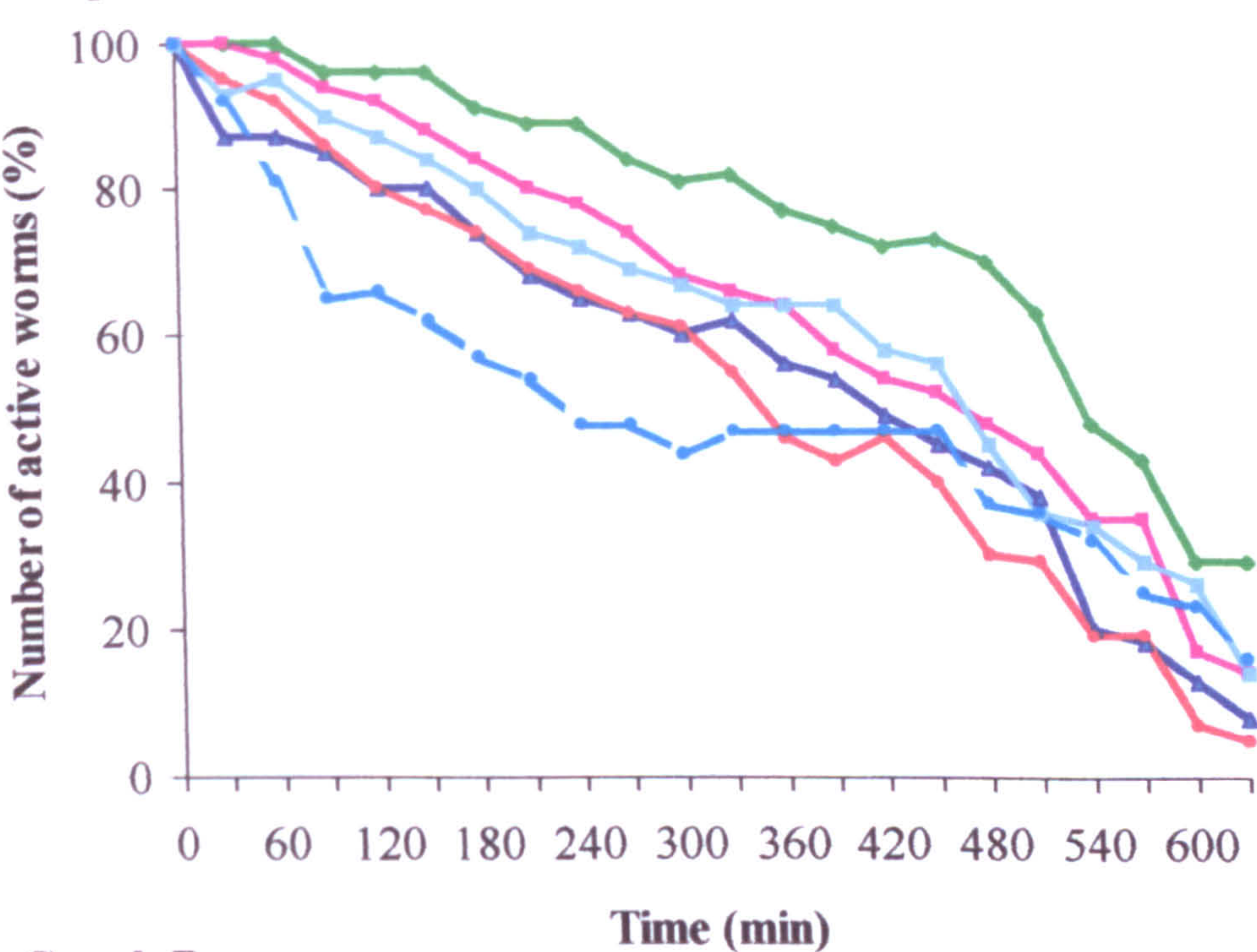
**Graph B** Effect of increasing concentrations of eQT in HBSS containing 0.1% (w/v) PEG on the survival of adult *N. brasiliensis* worms *in vitro*

Legend for Graph 6.3 A & B

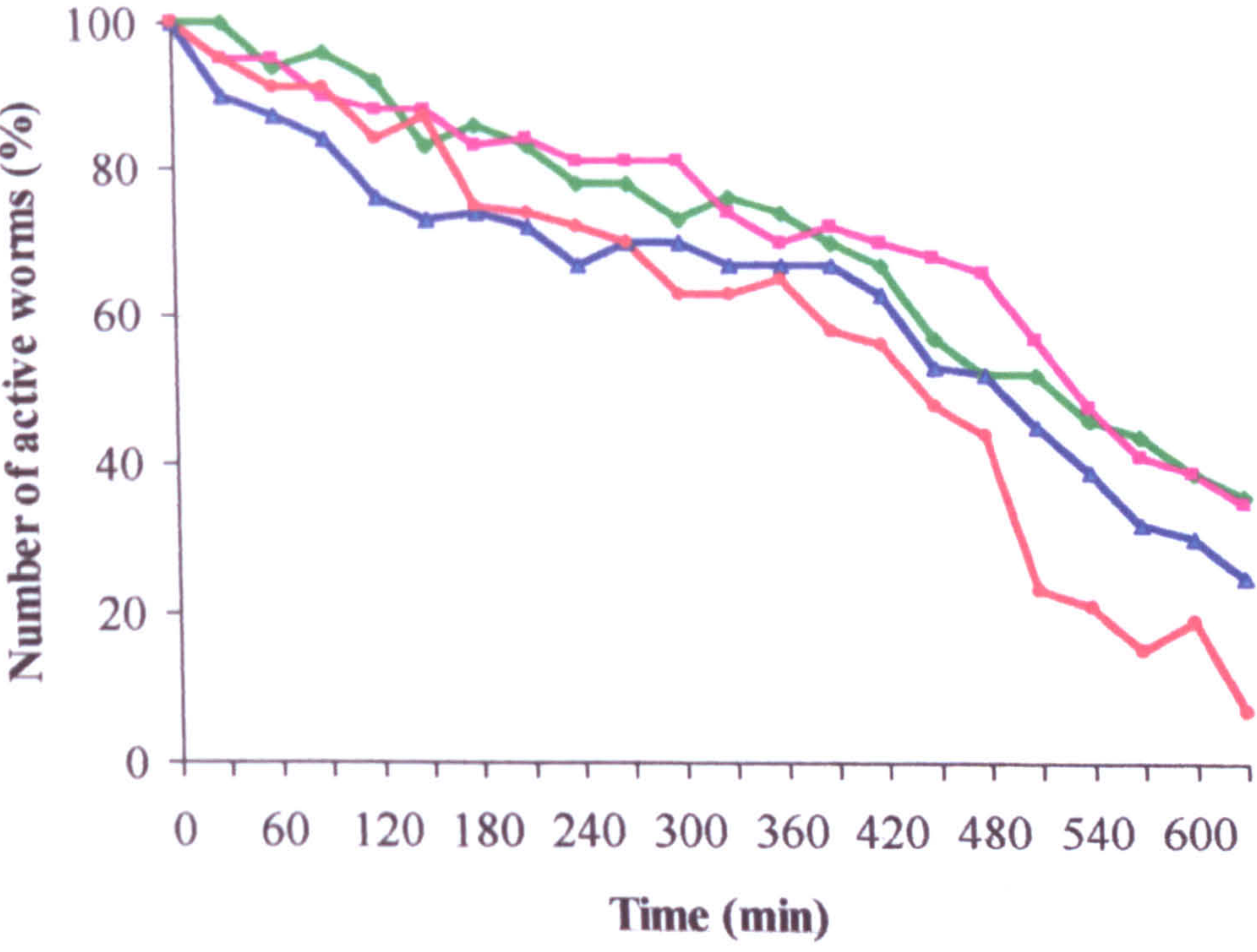




**Graph A**



**Graph B**





**Table 6.7** Time for 50% of adult *N. brasiliensis* worms incubated in varying concentrations of quebracho tannin (eQT) in HBSS alone or HBSS containing 0.1% PEG (w/v) to die.

| eQT concentration<br>(%, w/v) | 50% mortality<br>(min) | standard error | eQT concentration<br>(%, w/v) + 0.1% PEG (w/v) | 50% mortality<br>(min) | standard error |
|-------------------------------|------------------------|----------------|--|------------------------|----------------|
| 0.00                          | 534                    | 42             | 0.00   | 523                    | 79             |
| 0.01                          | 450                    | 79             | 0.01   | 541                    | 42             |
| 0.05                          | 385                    | 26             | 0.05   | 456                    | 15             |
| 0.125                         | 357                    | 38             | 0.125  | 413                    | 33             |

## 6.4 PRELIMINARY DISCUSSION

The aim of the studies reported in this chapter was to attempt to determine the mechanism through which quebracho tannin reduces the intensity of small intestinal nematode infection.

The results from the rat trial involving normal and immune-suppressed rats showed that the reduction in faecal egg counts was not significantly altered by immune-suppression, indicating that dietary eQT reduced faecal egg counts equally effectively in the immune-suppressed animal. This suggests that the mechanism through which dietary quebracho tannin reduces nematode infection is not through the up-regulation of the immune system of the host. This is also supported by data reported in Chapter 5 from lambs infected with *T. colubriformis* where no elevations in the immune responses lambs (ELISA and eosinophilia data) were observed from tannin-fed animals. However, it was assumed that injecting the animal with hydrocortisone sodium succinate had suppressed the immune system. The degree of suppression of the immune system was not quantified during the trial.

The results from the rat trial involving the infection of rats with either *N. brasiliensis* or *T. spiralis* indicated that dietary eQT had little effect on reducing the worm burden of mucosal inhabiting worms (eg. *T. spiralis*), whereas lumen dwelling nematodes (eg. *N. brasiliensis*) were susceptible. Worm burdens from *N. brasiliensis* infected rats were variable. Individual animal data beyond 2 standard deviations from the mean were removed before statistical analysis, as infection apparently was not uniform. The recovery of adult *N. brasiliensis* worms from the gut prior to the 'spontaneous' cure mechanism is generally expected to be approximately 50% of the number of infective larvae administered (Ash *et al.*, 1985; Lunn *et al.*, 1988). Those that fail to reach the intestine are mostly lost during the first day, either at the site of injection or during the course of migration (Jarrett *et al.*, 1968). The number of adult *N. brasiliensis* worms recovered on day 5 p.i. was approximately 50% of those administered indicating the viability of the worms was not responsible for the



variation in worm recovery. Therefore, the variability is most likely to be due to *N. brasiliensis* L<sub>3</sub> not being uniformly suspended in PBS for injection or the injected volume of PBS varied between animals. Once the results were adjusted for outliers the reduction in *N. brasiliensis* worm burden from tannin-fed rats was in agreement with data from chapter 3. The results from the present rat trial suggest that dietary eQT may be directly toxic against the worm and is less likely to influence infection by promoting the host immune response (supported by data from the immune suppression rat trial and sheep data).

The data generated from the *in vitro* experiments help to further confirm the hypothesis that quebracho tannin may be directly toxic to the worms. Adult *N. brasiliensis* worms incubated in quebracho tannin-containing media die more rapidly than those maintained in HBSS (control media). Worms incubated in quebracho tannin appeared to display increased activity possibly indicating that quebracho tannin is irritant to the worm. In addition, these worms tended to develop a dark line down the centre of the body, possibly due to ingestion of quebracho tannin by the worm resulting in the staining of the gut. Both forms of quebracho tannin were capable of reducing worm survival. This removes the speculation that the reduction in worm burdens and faecal egg counts of animals fed eQT may have been due to chemical residues remaining from the extraction process rather than the presence of condensed tannins. Interestingly, the death of worms incubated in HBSS containing quebracho tannin was much more rapid for those worms incubated in the untreated tannin (uQT) compared to the extracted form (eQT). This indicates that, while the condensed tannin concentration of uQT is only 80% of that in eQT, the activity of the condensed tannins in uQT appears to be greater, possibly suggesting that the chemicals used in the extraction process may have reduced the activity of quebracho condensed tannins. Incubating worms in HBSS containing eQT and PEG prevented any acceleration of worm death when the PEG to eQT ratio was 10:1. PEG binds to and precipitates condensed tannin (Jones & Mangan, 1977) making it inert. As the ratio of eQT to PEG increased, the deleterious effects of eQT on the worm increased. The attainment of 50% mortality of worms incubated in eQT and PEG was slower than when compared to worms incubated in the same concentration of eQT alone.

The results gained in these studies can be summarised as follows. The inclusion of quebracho tannin in low protein diets fed to rats is unlikely to reduce *N. brasiliensis* infection by initiating anti-worm immune responses. *In vitro* experiments indicate that the presence of quebracho tannin reduces the survival of adult *N. brasiliensis* worms. Unlike lumen-dwelling worms, there appears to be no reduction in mucosal-inhabiting worms established in tannin-fed rats. These results suggest that direct contact between the nematode and quebracho tannin is required to cause a reduction in the worm burden, and that quebracho tannin is likely to be exerting a direct toxic effect against the worm.



## 7 GENERAL DISCUSSION

### 7.1 INTRODUCTION

The main objective of the work described in this thesis was to determine the effect of including low concentrations of condensed tannin in the diet of rats and sheep on the extent of small intestinal nematode infection. This was primarily focused on animals maintained on low planes of nutrition, with quebracho tannin being used as a model condensed tannin. Tropical and subtropical regions are characterised by low primary production and forage quality (Degen *et al.*, 1997). The climates of these areas generally favour the development and survival of animal parasites, increasing the likelihood of host exposure to infective larvae. Animals maintained on low planes of nutrition are more susceptible to the ravages of internal parasitic disease compared to animals in temperate regions (Waller, 1997). It has been widely reported that production losses and mortality rates due to internal parasites are lower in animals fed on higher planes of nutrition (Poppi *et al.*, 1986; Abbott *et al.*, 1988; Bown *et al.*, 1991b; Kyriazakis *et al.*, 1994; van Houtert *et al.*, 1995a,b, 1996). Anthelmintics are routinely administered to remove parasite burdens. However, the use of anthelmintics to control disease, particularly in tropical and subtropical areas, is often not feasible. Anthelmintics are either unaffordable, often of inferior quality that they are not effective, or their continued over-use and mis-use are leading to a state of total chemotherapeutic failure (Waller, 1997). There is an increasing need for alternative strategies to control parasitic disease that are economically and environmentally sustainable. Possible alternatives include grazing strategies, worm vaccines, copper therapy, biological control, supplementary feeding, breeding approaches, ethno-veterinary preparations and condensed tannins (Waller, 1999).

## 7.2 SUMMARY OF FINDINGS

As stated above, the work carried out for this thesis involved assessing the effect of including a model condensed tannin, quebracho, in animal diets on the establishment and persistence of small intestinal nematode infections. Previous work carried out at the University of Nottingham indicated that dietary quebracho tannin may up-regulate the immune system, giving the host a greater capacity to withstand parasitic infection (see section 1.5.1, chapter 1). Similarly, improving the nutritional status of the host (e.g. increasing dietary protein concentration) can accelerate the maturation of the host immune response to internal parasites (Wan *et al.*, 1989). Animal diets were formulated to contain either high or low dietary protein concentrations (using as far as possible the same dietary ingredients) in order to investigate the effect of including dietary quebracho tannin in diets of different protein concentration. The interaction and possible additive effect of high protein and dietary quebracho tannin on small intestinal nematode infection were also investigated. Throughout the studies reported in this thesis, a number of haematological and serological measurements were taken to determine the immune status of the host animal.

Preliminary data obtained using the *N. brasiliensis*-rat model (chapter 3) established that the dietary inclusion of quebracho tannin reduced *N. brasiliensis* infection in rats. Predominantly, the trials were carried out using commercially available quebracho tannin that had been extracted to improve solubility and colour for use in the leather industry. To confirm that any effects of dietary quebracho tannin were due to the presence of the condensed tannins themselves rather than chemical residues remaining in the compound, two rodent trials were carried out using the extracted quebracho tannin (eQT) along with a natural quebracho tannin, which had not been chemically treated (uQT). Both tannin types proved to be effective at reducing *N. brasiliensis* infection. As anticipated, feeding a high protein diet reduced *N. brasiliensis* infection compared to feeding the low protein diet. However, there appeared to be no additional reduction in nematode infection when the high protein-tannin diet was fed. Work then progressed to determine whether dietary quebracho tannin was also effective at reducing *Trichostrongylus colubriformis* infection in



lambs (chapter 4). Animals maintained on poor planes of nutrition are generally more susceptible to parasitic infection, and, as only a limited number of lambs were used in this study and no additional benefits were observed by feeding high protein-tannin diets to rats, the lambs were fed at a restricted intake to gain approximately 100 g bodyweight/day. The results presented in chapter 4 demonstrated that dietary quebracho tannin was also effective at reducing *T. colubriformis* infection in lambs. Further to this, a larger sheep study was then carried out. The experiment described in chapter 5 investigated whether, to be effective, prior exposure to dietary quebracho tannin was required before nematode infection, or whether dietary quebracho tannin could reduce parasite burden in lambs with established infections. It was postulated that if dietary quebracho tannin reduced worm burdens through the stimulation of the host immune system, tannin may be required in the diet before nematode infection. The inclusion of dietary quebracho tannin in lambs with established infections was designed to determine the capacity of quebracho tannin to induce rapid effects to reduce nematode infection. In addition, the interactions of quebracho tannin with high and low dietary protein concentrations were also determined. The results showed that quebracho tannin reduced *T. colubriformis* infection in lambs fed low protein diets whether included in the diet before infection or once the infection was established. The reduction in *T. colubriformis* infection by dietary quebracho tannin was similar to the reduction seen after feeding the high protein diet. However, the inclusion of quebracho tannin in the high protein diet had no effect on the worm burden with faecal egg counts being similar to low protein-fed control lambs. This led to further investigations, reported in chapter 6, to determine the mechanism through which quebracho tannin reduced parasitic infection. Immune-suppressed rats fed the tannin diet had similar faecal egg counts and *N. brasiliensis* worm burdens recovered on day 7 p.i. compared to normal tannin-fed rats. This suggests that quebracho tannin is unlikely to be acting through altering the immune-responsiveness of the host. Thus, it was postulated that quebracho tannin may be directly toxic against the worm i.e. acting as an anthelmintic. In addition the effectiveness of dietary quebracho tannin against lumen-dwelling worms (*N. brasiliensis*) and mucosal-inhabiting worms (*Trichinella spiralis*) establishing in the rat small intestine were investigated. The results showed that unlike *N. brasiliensis* worms, *T. spiralis* worms were not affected by dietary quebracho tannin, suggesting



that direct contact between quebracho tannin with adult nematodes is necessary to promote worm death. Finally, *in vitro* experiments monitoring the survival of *N. brasiliensis* in varying concentrations of quebracho tannin were carried out. These results suggested that quebracho tannin may have anthelmintic properties against small intestinal nematodes that are in direct contact with the compound.

### 7.3 PERFORMANCE OF THE PARASITE-INFECTED ANIMAL

Symptoms of subclinical parasitic infection include inappetence, reduced liveweight gain and depressed wool and milk production (Poppi *et al.*, 1990). Inappetence is a common symptom of endoparasitic infection and further exacerbates the nutritional demand of the parasitised animal (Kyriazakis *et al.*, 1998). Inappetence was not observed during the infection period with the rat (chapters 3 & 6) or lambs that were fed at a restricted intake (chapter 4). The duration of *N. brasiliensis* infection in the rat is very short (approximately 10 days) and any feed refusals would only have been apparent over a short time period. Except for the first trial reported in chapter 3 where daily feed intakes were measured, feed intakes were recorded every two or three days and this may have prevented inappetence being observed. The lambs used in the sheep study reported in chapter 4 were fed at a restricted intake to gain approximately 100g bodyweight/day. Feed was generally consumed within one hour, and, although no feed refusals were observed by infected animals during the infection period, feed frequently took longer to be consumed, often over several hours. Data from Steel *et al.* (1980) suggest that if feed had been available *ad libitum* to the sheep in the study reported in chapter 4, feed refusals would probably have been observed. In the second study the lambs were fed just below *ad libitum* (4% bodyweight) and significant feed refusals were observed (approximately 30% feed refused) once the infection had established in the small intestine (approximately 3-4 weeks after initial infection).

Reductions in growth rates following infection were not clearly observed in the rat due to the short time period of infection. Reduced growth rates following trickle



infection of *T. colubriformis* were seen in the second sheep study reported in chapter 5 when compared to uninfected lambs fed the same dietary protein concentration. Increasing the dietary protein concentration fed to infected lambs from 97 g CP/kg to 222 g CP/kg improved daily liveweight gain to that of low protein-fed uninfected controls, providing further evidence that additional dietary protein reduces production losses associated with parasite infection (Poppi *et al.*, 1986; Abbot *et al.*, 1988; Bown *et al.*, 1991b; Kyriazakis *et al.*, 1994; van Houtert *et al.*, 1995a, b, 1996).

## 7.4 EFFECT OF DIETARY CONDENSED TANNINS ON PARASITIC INFECTION AND NUTRIENT SUPPLY

Condensed tannins are a diverse group of compounds that vary widely in chemical structure and degree of polymerisation (Horigome *et al.*, 1988). Thus, results gained for one type of condensed tannin can not necessarily be applied to different tannin types. In addition, the composition and structure of dietary protein is an important determinant of the tannin-protein interaction (Hagerman, 1989). Therefore the action of condensed tannin in natural forage, such as lotus, may be different to that of quebracho tannin included in a basal diet. The specificity of the tannin-protein interaction is further discussed in section 7.4.2.

### 7.4.1 Effect of condensed tannins on parasitic infection

The results presented in this thesis have involved individually housed animals which have been artificially infected with small intestinal nematodes and fed a basal diet in which the condensed tannin, quebracho, was included. In the literature available, relatively few papers have been published reporting the effects of dietary condensed tannins on parasitic infection. Of those available, the experimental design of the studies has often been limited to sheep consuming natural forages that contain condensed tannins compared to sheep grazing a different forage where the condensed

tannin concentration is negligible. Work published by Niezen *et al.* (1993) appears to be the first indicating a role of condensed tannins in the reduction of parasitic infection. Feeding the condensed tannin-containing forage sulla (100-120 g condensed tannin/kg dry matter (DM)) reduced faecal egg counts and also improved liveweight gains compared to those grazing lucerne (negligible levels of tannin) (Niezen *et al.*, 1995; 1998a). These authors postulated that the presence of condensed tannins protected dietary protein from rumen degradation and the subsequent increase of post-ruminal protein availability primarily mediated these effects. In the sheep studies reported in this thesis liveweight gains were not improved by the inclusion of quebracho tannin to the diet. The nitrogen content of the diets were equal (comparing diets of the same dietary protein concentration with and without quebracho tannin), while studies comparing two different forages often resulted in different nutrient intakes. A subsequent study by Niezen *et al.* (1998b) reported superior growth in lambs fed *Lotus pedunculatus* (approximately 50 g condensed tannin/kg DM) compared to those fed ryegrass (condensed tannin-free). In this study the nitrogen intake of lotus-fed lambs was approximately 3-fold greater than ryegrass-fed lambs, hence, any improvement in animal growth may be primarily due to additional nitrogen availability rather than any effect of condensed tannins in the forage. Thus, direct comparisons between sheep fed a condensed tannin-containing forage and those fed a tannin-free forage need to be considered critically in relation to the different nutritive values of the different forages.

Feeding quebracho tannin in a low protein diet reduced *T. colubriformis* infection in the sheep trials presented in chapters 4 & 5. In the literature, the effect of condensed tannins on parasite burden in the studies reported by Niezen *et al.* (1994, 1995, 1998a,b) and Robertson *et al.* (1995) appears to vary between different condensed tannin-containing forages and different parasite species. While growth rates were generally improved in infected lambs grazing condensed tannin-containing forages (sulla and lotus species) reductions in faecal egg counts and worm burdens varied. Niezen *et al.* (1995) reported approximately 50% reduction in eggs per gram (EPG) and worm burdens from sheep grazing sulla (condensed tannin 100-120 g condensed tannin/kg DM) but not lucerne (condensed tannin negligible). However, while the numbers of adult *Trichostrongylus* sp. recovered from the gut were reduced in sheep



grazing sulla, *Teladorsagia (Ostertagia) circumcincta* burdens were unaffected. Reductions in EPG were seen from naturally infected sheep grazing sulla, however, lotus species (*Lotus corniculatus* or *L. pedunculatus*) had no measurable effect on EPG when compared to condensed tannin-free forages (Robertson *et al.*, 1995; Niezen *et al.*, 1998a). In the literature reported above variation in total daily faecal output was not considered. During the rat trials (chapter 3 & 6) dietary quebracho tannin significantly increased faecal output, and hence caused a dilution effect when EPG was calculated. An elevation in faecal output from lambs grazing lotus was observed by Niezen *et al.* (1998b). During this study indoor housed sheep were infected with a single dose of *T. colubriformis* and *T. circumcincta* and fed either ryegrass (condensed tannin-free) or *L. pedunculatus* (56 g condensed tannin/kg DM). EPG were reduced by approximately 50% in the lotus-fed lambs, however, this apparent reduction in EPG was negated when total daily faecal egg counts were estimated. Niezen *et al.* (1998b) determined faecal output from the DM content of faeces collected at one time point (day 28 post infection (p.i.)) and DM intakes, estimated on days 24-25 p.i., when lotus-fed lambs consumed double the DM of ryegrass-fed lambs. It was estimated that lotus-fed lambs shed 24% more eggs than lambs fed ryegrass, when corrected for daily faecal output. The worm burdens recovered from the sheep on days 28 or 29 p.i. showed that lotus was effective at reducing the worm burden of *T. circumcincta* with the male:female ratio being increased. In ryegrass-fed sheep the establishment of *T. circumcincta* was estimated to be approximately 50% of the infective dose, whereas approximately 30% established in lotus-fed sheep. No significant effects were reported with *T. colubriformis* (Niezen *et al.*, 1998b), whereas quebracho tannin was effective at reducing *T. colubriformis* infection in lambs used in the two sheep trials reported in this thesis. Thus, the variation in response of the animal and infection level after feeding condensed tannin-containing forages suggests that the effectiveness of condensed tannins may be concentration- or type-dependant and that different parasites may be susceptible to different types or concentrations of condensed tannins. The location of the parasite may also alter the effectiveness of condensed tannins as described in section 7.6.3. The above literature and the results in this thesis suggests that the small intestinal nematode *T. colubriformis* appears more susceptible to sulla (Niezen *et al.*, 1995) and also quebracho tannin, while the

abomasal nematode *T. circumcincta* appears more susceptible to *L. pedunculatus* (Niezen *et al.*, 1998b). Evidence to support differences in the response of the animal to different condensed tannin structures are discussed in the following two subsections.

#### **7.4.2 Effect of condensed tannins on rumen metabolism and post-ruminal supply**

While the effect of quebracho tannin on rumen metabolism and post-ruminal supply was not investigated during the studies reported in this thesis, the ability of condensed tannins to prevent rumen degradation of dietary protein and hence increased post-ruminal protein supply forms the basis of one hypothesis of how condensed tannins reduce parasite infection (Niezen *et al.*, 1993). The following section discusses how different condensed tannin structures and variation in basal diet can alter the degradation of dietary protein in the rumen.

Forages containing moderate concentrations of condensed tannins (25-50 g/kg DM) can be beneficial when fed to sheep due to the protection of dietary protein from microbial degradation in the rumen (see section 1.3.4.2, chapter 1). These benefits have been observed after feeding lotus, either *L. corniculatus* (approximately 25 g condensed tannin/kg DM) or *L. pedunculatus* (approximately 50 g condensed tannin/kg DM), with benefits generally being greater for *L. corniculatus*. While the advantages of improved post-ruminal protein supply are not confined to lotus, the literature tends to focus on these two plant species. It is possible that the chemical structure of condensed tannins in the lotus species may be more important than the concentration of condensed tannins in the forage (Barry & McNabb, 1999). Lotus tannins differ in their major proanthocyanidin-type subunits, *L. corniculatus* consists predominantly of epicatechin (Foo *et al.*, 1996) while in *L. pedunculatus* epigallocatechin prevail (Foo *et al.*, 1997). Thus *L. pedunculatus* may be less beneficial than *L. corniculatus* possibly due to the condensed tannins in *L. pedunculatus* not releasing all the protein in the small intestine, increasing



endogenous secretions or inactivating digestive enzymes. In comparison, the structure of condensed tannins of quebracho is different to that of lotus, being primarily profistidinins (Streit & Fengel, 1994). While lotus species are generally advantageous to the animal, the inclusion of 50 g quebracho tannin/kg diet tends to have deleterious effects, including depressed microbial activity, reductions in protozoal numbers and histological changes of the small intestine (Salawu *et al.*, 1997, 1999; Dawson *et al.*, 1999). High concentrations of condensed tannins in *L. pedunculatus* (approximately 100 g/kg DM) also depress rumen function, reducing the digestion of readily fermentable carbohydrate and hemicellulose possibly by the precipitation of the extra-cellular microbial enzymes involved in the degradation of the components (Barry & Manley, 1999). However, the negative effects on rumen function were counteracted by increased post-ruminal non-ammonia nitrogen flow and nitrogen retention (Barry & Manley, 1984; Barry *et al.*, 1986b). Ulyatt & Egan (1979) found no evidence of impaired rumen digestion of carbohydrates after feeding sheep sainfoin (60 g condensed tannin/kg DM) compared to perennial ryegrass and white clover. Sainfoin tannins have a lower astringency and approximately three-fold higher molecular weight than lotus tannins (Jones *et al.*, 1976). The difference in molecular weight and reactivity of sainfoin condensed tannins may explain the absence of impaired rumen function (Barry & Manley, 1984). This further supports that different condensed tannins have different effects and also magnitude of effects on animals. Protein structure also plays an important role in the affinity of proteins for condensed tannins. Hagerman & Butler (1981) concluded that proteins with low molecular weights (below 20 000 MW) generally have low affinities for tannin, except for proline-rich protein. Proteins that have a high affinity for tannin generally have an open structure that is readily accessible for hydrogen bonding. Proteins with compact globular structures, where hydrogen bonding with tannin can not be readily accomplished, have a low affinity for tannin (Hagerman, 1989). Thus, the inclusion of quebracho tannin in a basal diet may be different to results gained by feeding lotus species that naturally contain condensed tannins. Condensed tannins may be less effective in increasing duodenal nitrogen flow when the basal diet contains lower quantities of rumen degradable protein, or protein that has a more compact structure. When large quantities of rumen degradable protein are available, the tannin-protein complex that is formed in the rumen would reduce the potential capacity of



condensed tannins to actively inhibit rumen function. Barry & Manley (1986) estimated that 90% of total condensed tannins in lotus were bound to plant protein (unless the binding capacity of the system is exceeded, above approximately 90 g condensed tannin/kg DM), leaving only 10% condensed tannins with the potential to inactivate microbial enzymes. Hence, if dietary quebracho tannin has less potential to bind to dietary protein, a greater proportion of free condensed tannins could enter the rumen and induce greater detrimental effects on rumen function.

Condensed tannins are known to have bacteriostatic and bactericidal effects (Henis *et al.*, 1964). Section 1.3.4.1 (chapter 1) reviews some of the literature where condensed tannins have had deleterious effects on rumen microorganisms. Makkar *et al.* (1995a, b) suggested that rumen microorganisms may adapt to dietary tannin with prolonged feeding by producing enzymes capable of degrading tannins. However, Dawson *et al.* (1999) did not observe any adaptation (improved nutrient digestibility) in sheep fed 50 g quebracho tannin/kg diet for 6 weeks compared to results gained after feeding the diets for 2 weeks. Tannin-tolerant bacteria have, however, been identified in East African ruminants adapted to forage containing condensed tannins (Odenyo & Osuji, 1998). Isolates of anaerobic bacteria characterised as *Selenomonas* species were able to grow in the presence of 8 g of purified condensed tannins/l. Growth at higher concentrations was not tested (Odenyo & Osuji, 1998). Similarly, anaerobic bacteria isolated from ruminal fluid of goats fed condensed tannin forages remained morphologically unchanged when incubated in media containing 4 g quebracho tannin/l (Nelson *et al.*, 1995) and grew in media containing at least 25 g *Acacia* condensed tannins/l (Booker *et al.*, 1994). Nelson *et al.* (1998) concluded that the presence of tannin-tolerant bacteria was not affected by climate, geography, or host animal (present in Sardinian sheep, Honduran and Colombian goats, white-tailed deer and Rocky Mountain elk), but was restricted to animals that had previously consumed a tannin diet, suggesting adaptability of micro-organisms.



### 7.4.3 Post-ruminal effects of condensed tannins

Condensed tannins protect dietary protein from rumen degradation by forming insoluble complexes with the protein. Once the complex is exposed to gastric (pH 2.5-3.5) and pancreatic (pH 8-9) secretions, dissociation occurs and the protein is released increasing the supply of undegraded protein entering the duodenum (D'Mello, 1992). However, the fate of the free condensed tannins in the small intestine remains obscure. It might be expected that free condensed tannins would inhibit digestive enzymes in the small intestine and have activity against the gut wall, as observed in the monogastric animal (see section 1.3.3.3, chapter 1). Again, different condensed tannins appear to exert different effects. Santidrian (1981) reported a reduced intestinal absorption of D-glucose, D-galactose and L-leucine after feeding faba beans to rats. When included in porcine diets, van Leeuwen *et al.* (1995) recorded a depression in aminopeptidase activity but no alteration in sucrase-isomaltase activity, indicating a differential specificity for different enzymes. Enzyme inhibition has been shown to generally be less severe *in vivo* compared to results from *in vitro* studies (Oh & Hoff, 1986) suggesting that salivary proline-rich proteins (PRPs) play an important role in tannin detoxification. In addition to the complex formed between condensed tannin and dietary protein reducing protein availability, any reduction in digestive enzyme activity will further reduce nutrient availability to the animal, which could explain the depressed growth rates seen in the rat trials presented in this thesis.

The results from the rat trials in chapter 3 showed that the inclusion of dietary quebracho tannin reduced *N. brasiliensis* infection. Worm burdens recovered just post-establishment in the rat small intestine (day 5 p.i.) indicated that the inclusion of quebracho tannin reduced the number of worms establishing in the small intestine. The plane of nutrition does not appear to affect the establishment of infective larvae, particularly with respect to ruminants (Bown *et al.* 1991b; van Houtert *et al.*, 1995a, b & 1996; Coop, 1998) but also rats (Lunn *et al.*, 1988). Accordingly, the numbers of *N. brasiliensis* recovered from the rat on day 5 p.i. were similar irrespective of dietary protein concentration. However, Ash *et al.* (1985) recovered significantly

greater numbers of *N. brasiliensis* from protein-malnourished rats (2g protein/kg) compared to rats fed 16g protein/kg. In addition, the duration of worm infection was extended in the protein-malnourished rat. This unexpected effect of dietary protein on nematode establishment rates may have been a response of the severe protein restriction enforced on these rats, whereas the protein restriction in the rat trials presented in this thesis was only moderate. Niezen *et al.* (1993, 1995) suggested that the reduction in EPG and worm burdens of sheep fed condensed tannin-containing forages was most likely to be a consequence of improved post-ruminal protein supply. However, the capacity of dietary quebracho tannin to reduce *N. brasiliensis* infection in the rat i.e. non-ruminant, irrespective of protein status, indicates that the quebracho tannin reduces worm infection through a different mechanism, possibly through promoting a host immune response or exerting a directly toxic effect against the worm (see section 7.6).

## 7.5 EFFECT OF DIETARY QUEBRACHO TANNIN AND PROTEIN CONCENTRATION ON THE PARASITE-INFECTED ANIMAL

Results from the trials reported in this thesis show that tannin-fed animals tend to pass a greater quantity of faeces/day compared to control-fed animals. An increased daily faecal output would cause a dilution effect on the number of nematode eggs passed per day when determined on a fresh faecal gram basis (EPG). Hence, faecal egg counts were expressed as total daily output, except during the sheep study where lambs were fed a restricted intake (chapter 4), as total daily faecal output was not measured. Published results of faecal egg counts from tannin-fed animals do not generally make allowances for increased faecal output and caution has to be applied when comparing these results (see section 7.4.1). As anticipated, feeding high protein (H) diets to both rats and lambs significantly reduced total daily faecal egg output compared to low protein (L) fed animals. The inclusion of quebracho tannin in the low protein diet (L+QT) reduced faecal egg counts to similar concentrations as animals fed diet H. Faecal egg counts have been shown to have a good correlation with worm burdens for sheep and goats (Cabaret *et al.*, 1998). Therefore dietary quebracho tannin may have depressed egg counts through reducing the number of



worms present in the small intestine; this was shown by the reduction in *N. brasiliensis* worms establishing in the small intestine of tannin-fed rats (chapter 3). The inclusion of quebracho tannin in the high protein diet (H+QT) fed to rats did not have any additional effects compared to feeding diet H. Feeding quebracho tannin with high- or low-protein diets or feeding diet H alone to lambs in chapter 5 reduced faecal egg counts (EPG) compared to lambs fed diet L. However, the inclusion of quebracho tannin in the high protein diet had no effect on total daily faecal egg output compared to lambs fed diet L. One possible explanation for the lack of interaction between quebracho tannin and dietary protein concentration may be related to the binding capacity of quebracho tannin with dietary protein. This therefore suggests that dietary protein and quebracho tannin reduce worm burdens through different mechanisms.

In the rat trials reported in this thesis the inclusion of 40g quebracho tannin/kg diet reduced growth rate at both high and low dietary protein concentrations. This was presumably due to the formation of tannin-protein complexes, reducing dietary protein availability. The growth rate of rats fed H+QT generally tended to be greater than that of rats fed diet L, suggesting that protein availability was still greater even in the presence of quebracho tannin. The additional protein available to the H+QT fed rats, compared to those fed diet L, may have enabled the rats to have an enhanced immune response against the worm burden, greater than diet L-fed rats but less than rats fed diet H. Dietary quebracho tannin would have bound to some of the additional protein in diet H+QT, hence the reduced growth rate. However, some tannin 'activity' is likely to have remained as *N. brasiliensis* burdens were significantly reduced in H+QT and L+QT fed rats on day 5 p.i. compared to H and L-fed rats. Similar total daily faecal egg counts from rats fed diet H+QT to those from rats fed diet H or L+QT, suggest that the capacity of diet H+QT to reduce worm infection was equivalent to either feeding diet H or L+QT. Thus feeding quebracho tannin in a high protein diet did not have an additive effect at reducing worm infection. The reduction in total daily faecal egg counts by diet H+QT, compared to diet L, may have been a consequence of the combined partial enhancement of the immune response (greater than diet L but less than diet H) and the partial 'activity' of

quebracho tannin (additional dietary protein binding to some of the quebracho tannin and partially reducing tannin 'activity').

In the sheep studies, lambs fed diets H and L+QT had significantly reduced total daily faecal egg counts compared to lambs fed diet L. A significant reduction in mean EPG from H+QT-fed lambs compared to the diet L-fed lambs was also seen. However, the H+QT-fed lambs excreted a significantly greater quantity of faeces compared to any other dietary group. When mean total daily faecal egg counts were estimated using daily faecal output, H+QT-fed lambs and lambs fed diet L passed similar quantity of nematode eggs. The profile of total daily faecal egg counts showed that H+QT-fed lambs had a similar peak egg output with diet H- and L+QT-fed lambs (Figure 5.2, chapter 5). However, H+QT-fed lambs were unable to expel the worm burden and faecal egg counts remained elevated at the end of the 10 week infection period compared to the other infected dietary groups. This continued egg output was primarily responsible for the similar total daily faecal egg output with lambs fed diet L. Therefore the suggestion of a combined partial enhancement of the immune response and the partial 'activity' of quebracho tannin in H+QT diets (additional dietary protein binding to some of the quebracho tannin and partially reducing tannin 'activity') may remain appropriate to explain the apparent absence of nematode reduction in lambs fed H+QT. The binding of additional dietary protein to quebracho tannin could reduce tannin 'activity' and protein availability. In addition, the differences in the response of the rat and sheep to expelling the worm burden may be a factor of the different nematode species. Adult *N. brasiliensis* worms dwell in the small intestine while adult *T. colubriformis* worms establish in the intra-epithelium of the small intestine with the posterior of the worm often found in the lumen (Miller, 1984). The different proportions of the body of each worm exposed to digesta, and hence direct contact with quebracho tannin, may help explain the apparent increased sensitivity of *N. brasiliensis* compared to *T. colubriformis*.



## 7.6 MECHANISM OF ACTION OF QUEBRACHO TANNIN

The results from chapters 3, 4 & 5 suggest that dietary quebracho tannin did not reduce small intestinal nematode infection in sheep purely through an increase in protein availability to the host, and thereby elevating the immune response against the worm burden. Therefore it was postulated that dietary quebracho may have reduced nematode infection through one of three alternative mechanisms. The first was by the activity of tannins against the GI mucosa causing histological changes and increasing intestinal endogenous secretions. The latter two mechanisms considered suggest that quebracho tannin could induce the sensitisation of the host immune system, or had a direct toxic effect against the worm burden.

### 7.6.1 Increase of intestinal endogenous secretions by dietary quebracho tannin

It is possible that the inclusion of dietary quebracho tannin may have reduced worm burdens through increasing small intestinal endogenous secretions including hypersecretion of mucus. Increased mucus secretion and goblet cell hyperplasia are characteristic during the expulsion period of parasites from the digestive tract (Coop & Angus, 1975; Jackson *et al.*, 1983). Douch (1990) implicated the role of mucus secretion in worm rejection, where mucus can entrap larvae and prevent establishment. Alternatively, large quantities of mucus on the mucosal surface may inhibit the motility and feeding capacity of parasites (Miller, 1987), while the sloughing of mucosal epithelia is reported to be particularly effective at dislodging fourth-stage larvae and adult nematode worms (McClure *et al.*, 1992). Where resistance is lacking against worms, e.g. malnourished rats (Wells, 1963) and sheep (Dobson & Bawden, 1974), goblet cell hyperplasia is reduced. The role of goblet cells and mucus in the expulsion of adult *N. brasiliensis* in the mouse was shown where worm expulsion was accompanied by a two- to four-fold increase in goblet cell numbers and increased mucus secretion (Uber *et al.*, 1980). Both have been closely paralleled to the expulsion of *N. brasiliensis* (Lloyd & Soulsby, 1987) with

the interference of mucus function by the administration of drugs being shown to inhibit worm expulsion (Rothwell, 1989).

Evidence that dietary quebracho tannin may have increased endogenous secretions from the small intestine was obtained in nitrogen balance results reported in chapter 3. Total faecal nitrogen output was increased after feeding 40g quebracho tannin/kg diet. Elevations in total faecal nitrogen are likely to be a consequence of both impaired protein digestion and increased secretions of endogenous proteins. However the biological value of protein remained unaffected by dietary quebracho tannin indicating that protein metabolism and utilisation was unaffected by quebracho tannin, and the additional nitrogen recovered from the faeces of tannin-fed animals was most likely to have been contributed from endogenous secretions. During the rat trials it was noted that fresh faecal pellets from tannin-fed rats appeared to have a mucus membrane adhering to the pellet. This could correspond to the hypersecretion of mucus by the gut due to dietary quebracho tannin inclusion and contribute to the resultant elevation in faecal nitrogen. Ortiz *et al.* (1994) observed changes in the ileal mucosa, atrophy and shortening of villi and architectural distortion, after feeding faba beans containing condensed tannins to chicks; evidence of morphological damage to rat intestine was less apparent. Rats are capable of adapting to tannin-containing diets through the production of proline rich salivary proteins (PRPs; Jansman *et al.*, 1993). However, there is no evidence that chicks secrete these salivary proteins (Butler, 1989), possibly explaining the increased morphological damage seen by Ortiz *et al.* (1994). In contrast to the rat, domestic sheep do not produce tannin-binding salivary proteins (Austin *et al.*, 1989). Thus sheep may be more susceptible to GI tract damage and/or toxicity effects from dietary tannin as observed by Ortiz *et al.* (1993) in the chick. Dawson *et al.* (1999) has reported evidence of GI damage occurring in the sheep due to dietary condensed tannins. Focal surface epithelial damage and an increase of mucosal histiocytes after feeding 50g quebracho tannin/kg diet to ewe lambs were observed. Epithelial damage and ulceration were most apparent in animals slaughtered after the diet had been fed for 2 weeks suggesting it may be an early response feature and dependent on local concentration.



Mucus hypersecretion may play a further protective role against dietary tannins, either reducing tannin interaction with the mucosal surface or by forming inactive mucin-tannin complexes. Ortiz *et al.* (1994) observed increased goblet cell hypertrophy and hyperplasia in the ileal mucosa of both rats and chicks fed faba beans, resulting in a 3-fold increase in goblet cell numbers. The mucus membranes seen encapsulating faeces from tannin-fed rats were also observed from some tannin-fed lambs. Sheep faecal pellets were supported by what appeared to be a membrane, holding the faecal pellets together in an appearance like beads on a string. While it is unclear whether the inclusion of dietary quebracho tannin induced any morphological damage to the small intestine of animals used in the trials presented in this thesis it is possible that quebracho tannin may have induced mucus hypersecretion. Thus, the decrease in the worm burdens of rats and lambs fed diet L+QT may simply be a result of mucus hypersecretion induced by dietary quebracho tannin. The secretion of PRPs by the rat but not the sheep, would be expected to have bound quebracho tannin and reduced the potential 'activity', however it is not known to what extent pelleting of the sheep diet may have converted extractable tannin to protein-bound tannin and hence reduced the potential 'activity' of the tannin. Unpublished data by R. Parrinder, J.M. Dawson & P.J. Buttery showed that only 45% of quebracho tannin were extractable from the pelleted diet using a standard acid butanol assay while  $100 \pm 2\%$  quebracho tannin was recoverable before the diet was pelleted. The possible reduced activity of quebracho tannin due to the high temperatures involved in pelleting the diet may account for the apparent lack of reduction in total daily faecal egg output from lambs fed diet H+QT. The additional protein in the diet may have bound all the remaining 'active' sites on quebracho tannin leaving the tannin inert against the gut wall and the worm, with the possibility that additional protein availability was unable to enhance the immune response sufficiently to reduce the worm burden compared to lambs fed the low protein diet alone. Further work is required to establish the proportion of quebracho tannin that is no longer recoverable from both the high and low protein diets after pelleting.

### 7.6.2 Sensitisation of the host immune system by dietary quebracho tannin

After ingestion, infective trichostrongylid larvae ex-sheath and penetrate the gastrointestinal mucosa developing through the fourth stage larvae to become adult worms. Products of the worm are recognised by the host as non-self and initiate an immune response. Data by Dawson *et al.* (1999) suggested that quebracho tannin could be locally toxic to the surface epithelium of the small intestine with evidence that quebracho tannin was taken up by Peyer's patch M cells that may initiate an immune response. Eosinophilia is considered to be an important indicator of helminth infection and is associated with the expulsion of the parasite from the gut (Dawkins *et al.*, 1989), indicating a role in host resistance. The initiation of an immunological reaction is considered to be the trigger required for the elevation of peripheral blood eosinophils. Peripheral eosinophilia remained low in sheep fed control and tannin diets in both sheep studies (chapters 4 & 5) until the worms had established in the small intestine. Eosinophil counts began to rise from around the time of peak egg output in both sheep studies. The absence of any early elevation of eosinophils counts in tannin-fed lambs during the one month prior to nematode infection, and no significant increase following infection, suggests that quebracho tannin did not initiate a general immune response that resulted in the elevation of peripheral eosinophil concentrations. During the second sheep study (chapter 5) the immune responsiveness of the host against a foreign protein, ovalbumin, was quantified using ELISA. Infection, protein concentration or dietary tannin did not significantly influence the levels of circulating antibodies to ovalbumin. Further evidence indicating that dietary quebracho tannin does not appear to enhance an immune response was obtained from immune-suppressed rats (chapter 6). In this study dietary quebracho tannin was found to be equally effective in immune-suppressed and normal rats. However, the results from this trial need to be judged critically as explained in section 6.4, as it was not quantified whether the immune system was sufficiently suppressed in those rats injected with the immune-suppressant.



### 7.6.3 Direct toxicity of dietary quebracho tannin against the parasite

The hypothesis that quebracho tannin could be directly toxic against the worm burden is supported by data presented in this thesis. The mechanism through which this could occur remains somewhat unclear although ideas can be postulated. If quebracho tannin is directly toxic against the worm this could explain the reduction of *N. brasiliensis* worms at establishment and the depressed faecal egg counts observed when quebracho tannin was included in the low protein diet either at infection (rats, chapter 3) or once the nematodes had reached sexual maturity (lambs, chapter 5). This is further supported by data presented in chapter 6. The numbers of mucosal inhabiting nematodes, *Trichinella spiralis*, were not affected by the inclusion of quebracho tannin in the diet, whereas *N. brasiliensis* worm burdens were significantly reduced. It is possible that quebracho tannin was ineffective against *T. spiralis* due to the short time period that the larvae were exposed to gut contents before burrowing into the mucosa. In contrast *N. brasiliensis* worms are continually exposed to gut contents, feeding mainly on blood, tissue cells, but also intestinal contents (Smyth, 1994). Therefore ingestion of quebracho tannin, either from intestinal contents and/or that bound/incorporated into gut epithelial tissue would occur. The exposure of *T. spiralis* to quebracho tannin and the opportunity to ingest tannin would be limited and may explain the lack of reduction in worm burdens in tannin-fed rats.

The natural function of condensed tannins in cereals and legume seed is to play a role in the crop's defence mechanism reducing herbivore consumption and susceptibility to attack by fungi and pests (Jansman, 1993). Some plants have also developed strategies to reduce attack from plant parasitic nematodes. Leaf extracts from wild sage have proved lethal to root-knot nematode larvae, but the effective agent/compound against the nematode was not identified (Chandel & Metha, 1990). Earlier work by Taylor & Murant (1966) observed reduced populations of plant parasitic nematodes in the soil after raspberry canes were incorporated. The toxic substance in raspberry cane was suggested to be tannin. Quebracho and mimosa tannin (condensed tannin) and to some degree hydrolysable tannins were shown to

reduce plant parasite numbers (Taylor & Murant, 1966). Not only have plant extracts, including tannins, reduced numbers of plant parasitic nematodes, Lorimer *et al.* (1996) reported reductions in the motility of larval *T. colubriformis*. One plant species analysed, *Phyllocladus aspleniifolius* var. *alpinus*, which inhibited *T. colubriformis* larval motility was known to contain polyphenolics (Foo *et al.*, 1985). Removal of polyphenolics gave *P. aspleniifolius* var. *alpinus* extracts greatly reduced inhibition of larval migration activity. Finally, eucalyptus species have proved effective against the abomasal nematode *Haemonchus contortus* but not *Teladorsagia* spp. (Bennet-Jenkins and Bryant, 1996). In addition to some condensed tannins having toxic effects against some plant and animal nematodes, they also possess some bacteriostatic and bacteriocidal effects as discussed in section 1.3.4.1 (chapter 1).

The results of the *in vitro* experiments further support a direct toxicity effect of quebracho tannin against nematodes that are exposed to the compound. Concentrations as low as 0.01% (w/v) quebracho tannin accelerated the mortality of adult *N. brasiliensis* worms *in vitro*. The addition of PEG (MW 3500) to the media reduced the rate of mortality. PEG binds to condensed tannins leaving them inert (Jones & Mangan, 1977). The reduction in mortality suggests that not all the condensed tannin in the quebracho compound were fully bound when a ratio of PEG to quebracho tannin of 2:1 was used. When a ratio of 10:1 (PEG:quebracho tannin) was used, no acceleration of worm mortality was seen compared to worms incubated in control media. Yu *et al.* (1995) found that a PEG-to-tannin ratio of 2:1 was sufficient to maximise protein displacement from the tannin-protein complex formed from condensed tannin present in cottonseed. Miller *et al.* (1997) established that the PEG to condensed tannin ratio of 0.7:1 (w/w) was sufficient to precipitate 77% of mulga condensed tannins. This has been established as the maximum quantity of mulga condensed tannins that can be precipitated by PEG (Pritchard *et al.*, 1992). This implies that either a greater PEG-to-tannin ratio is required for quebracho tannin, that tannin activity of quebracho may not be entirely removed by binding to PEG, or that components other than condensed tannin in quebracho may have anthelmintic properties. There is indication that quebracho tannin (both extracted, eQT, and untreated quebracho tannin, uQT) interferes with the parasite enzyme



glutathione transferase (GST) *in vitro*. The activity of the enzyme can be restored by the addition of polyvinylpyrrolidone (PVP). PVP, like PEG binds to condensed tannin and prevents further activity of tannin (Dr. B.B. Fakae, personal communication). GSTs are a widely distributed family of multifactorial proteins concerned with the detoxification of exogenously- and endogenously-derived toxic compounds (Brophy *et al.*, 1989). Helminths have limited detoxification enzymes, GST has been detected in a range of helminths and may be one of the major detoxification enzymes (as reviewed by Brophy & Barrett, 1990). The binding of GSTs to anthelmintics could alter the effective concentration of anthelmintics and may explain the relative sensitivity/resistance of these drugs. GSTs may either act as intracellular transport proteins potentiating anthelmintic effects or conversely, the enzymes may passively detoxify the compound (Brophy & Barrett, 1990). For example, Kawalek *et al.* (1984) correlated *H. contortus* resistance with increased GST levels. Therefore the removal of GST activity by quebracho tannin could make helminths more susceptible to other toxic compounds, possible host immune responses and/or anthelmintics. Brophy & Barrett (1990) concluded that the inhibition of GST could offer the possibility of combining immuno- and chemo-therapy due to the role played by GSTs protecting cells against immune-mediated lipid peroxidation. A recent study published by Athanasiadou *et al.* (1999) supports the hypothesis that quebracho tannins may be acting as an anthelmintic against small intestinal nematode infections. In this study, lambs were experimentally infected with a single large infective dose of *T. colubriformis*. On days 28 to 34 p.i. animals were given an oral drench of quebracho tannin (8% w/w of food intake). Faecal egg counts recorded during the drenching period were reduced by 50% in treated compared to control animals. Worm burden and *per capita* fecundity at slaughter (day 35 p.i.) were reduced by 30% in quebracho-drenched sheep. Whether the anthelmintic effects are a general feature of all condensed tannins or are exclusive to quebracho tannin remains to be established. However, it is likely that the mode of action of the anthelmintic effects of condensed tannins are quite different from that of conventional drugs that act via effects on nerve-muscle transmission.

## 7.7 CONCLUSIONS

Feeding low protein diets to monogastrics and ruminants can reduce their ability to mount an immune response to expel primary infections of nematodes from the small intestine. Increasing the protein content of the diet can improve the hosts' ability to expel the worm from the gut. The inclusion of 4 or 5% (w/w) quebracho tannin in low protein diets was effective at reducing small intestinal nematode infection in sheep and rats to levels observed in high protein-fed animals. The inclusion of quebracho tannin in high protein diets did not convey any further reduction of infection in the *N. brasiliensis*-rat model, while in the *T. colubriformis*-sheep model faecal egg counts were returned to levels observed in low protein fed sheep. Thus in tropical countries where infected animals are on a low plane of nutrition, the introduction of condensed tannins, either as a diet supplement or as a condensed tannin-containing forage, may be an alternative to increasing dietary protein concentration. The mechanism through which quebracho tannin reduced nematode infection appears to be through a toxic effect against the nematode. It appeared that the reduction of nematode infection occurred against worms that were in direct contact with quebracho tannin-containing digesta. This may limit the potential anthelmintic properties of dietary condensed tannins to those parasites that are present in the lumen of the gut and/or feed on intestinal contents, mucus and mucosal cells. Similarly, the anthelmintic properties of quebracho tannin may be exclusive to a specific condensed tannin structure.

## 7.8 FUTURE RESEARCH

This thesis has reported investigations of the effect of dietary quebracho tannin against GI nematode burdens in rats and sheep. Future work would logically progress to undertake research aimed at extending the work to the Tropics where naturally occurring forages containing condensed tannins could be fed to control parasitic infection. The use of condensed tannins to control internal parasitism would provide



an economic and environmentally sustainable alternative to anthelmintic drenches, and would also be advantageous for organic farming systems.

To achieve this, the elucidation of the mechanism through which quebracho tannin exerts anthelmintic effects against the burden would provide a basis from which to screen other plant species for their potential anthelmintic properties. An obvious point to begin from would be to obtain a purified sample of quebracho tannin, containing only condensed tannins, to unequivocally establish that the anthelmintic properties of quebracho tannin were due to the presence of condensed tannins in the compound. The undertaking of several biochemical, ultrastructural and metabolic studies of worms exposed to quebracho tannin would hopefully elucidate whether quebracho tannin causes the disruption of endogenous enzymes or induces hormonal imbalance or neural damage. Work would also need to continue to establish whether direct exposure to dietary tannin is necessary for any anthelmintic effects to be seen. This would enable predictions to be made as to which parasite species would be susceptible to condensed tannins. Once such information is available naturally occurring forage species could be screened for their potential anthelmintic properties and the susceptibility of different parasite species estimated.

In order to select a suitable forage an optimum concentration of condensed tannin that would exert maximal anthelmintic properties without causing adverse effects on animal production would need to be established. This could simply be obtained by undertaking a dose response experiment using different concentrations of quebracho tannin below and exceeding 50g quebracho tannin/kg used in the sheep trials in this thesis. In addition, the loss of quebracho tannin during pelleting would need to be determined, before possible naturally occurring plant species could be selected. Once suitable condensed tannin-containing forages had been selected, the sustainability of forages in a grazing system would then need to be considered. It may be necessary to grow the forage separately and introduce a cut and carry system to maintain the optimal condensed tannin concentration. The cut and carry system can be common practice in the Tropics where animals are tethered and feed is harvested and brought to them. Large animal trials could then be carried out under field conditions of

continuous multi-species infection to establish the effectiveness of the condensed tannin-containing forages selected at reducing a natural burden of intestinal parasites.



## REFERENCES

- ABBOTT, E.M., PARKINS, J.J. AND HOLMES, P.H. (1986) The effect of dietary protein on the pathogenesis of acute ovine haemonchosis. *Research in Veterinary Science* **20**, 275-289.
- ABBOTT, E.M., PARKINS, J.J. AND HOLMES, P.H. (1988) Influence of dietary protein on the pathophysiology of haemonchosis in lambs given continuous infections. *Research in Veterinary Science* **45**, 41-49.
- ADAMS, D.B., ANDERSON, B.H. AND WINDON, R.G. (1989) Cross-immunity between *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep. *International Journal for Parasitology* **19**, 717-722.
- AGRICULTURAL AND FOOD RESEARCH COUNCIL (1993) *Energy and Protein Requirements of Ruminants*. An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients. CAB International, Wallingford, UK.
- AHMED, A.E., SMITHARD, R. AND ELLIS, M. (1991) Activities of enzymes of the pancreas, and the lumen and mucosa of the small intestine in growing broiler cockerels fed on tannin-containing diets. *British Journal of Nutrition* **65**, 189-197.
- ALLEN, R.E. (1990) *The Concise Oxford English Dictionary of current English*. 8th Edition, Clarendon Press, Oxford.
- ANDERSON, N. (1982) Internal parasites of sheep and goats. In: *Sheep and Goat Production. World Animal Science, C1.*, pp. 175-192 (Ed. I.E. Coop) Elsevier Scientific Publishing Co. The Netherlands.
- ASH, C., CROMPTON, D.W.T. AND LUNN, P.G. (1985) Impact of *Nippostrongylus brasiliensis* (Nematoda) on the serum albumin and amino acid concentration of rats fed adequate or protein-deficient diets. *Parasitology* **90**, 157-168.
- ASQUITH, T.N. AND BUTLER, L.G. (1986) Interactions of condensed tannins with selected proteins. *Phytochemistry* **25**, 1591-1593.
- ATHANASIADOU, S., KYRIAZAKIS, I., JACKSON, F AND COOP, R.L. (1999) Effects of short-term exposure to condensed tannins on adult *Trichostrongylus colubriformis*. *Veterinary Record* In press.
- AUSTIN, P.J., SUCHAR, L.A., ROBBINS, C.T. AND HAGERMAN, A.E. (1989) Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. *Journal of Chemical Ecology* **15**, 1335-1347.

- BAE, H.E, MCALLISTER, T.A., YANKE, J., CHENG, K-J. AND MUIR, A.D. (1993) Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. *Applied and Environmental Microbiology* **59**, 2132-2138.
- BAILEY, K.M. (1991) Anthelmintic ineffectiveness in New Zealand 1989-1990. *Surveillance* **18**, 38-39.
- BANG, K.S., HAMILTON, A.S. AND SYKES, A.R. (1990) Effect of ostertagiasis on copper status in sheep: a study involving the use of copper oxide wire particles. *Research in Veterinary Science* **49**, 306-314.
- BARAHONA, R., LASCANO, C.E., COCHRAN, R., MORRILL, J. AND TIGEMEYER, E.C. (1997) Intake, digestion and nitrogen utilisation by sheep fed tropical legumes with contrasting tannin concentrations and astringency. *Journal of Animal Science* **75**, 1633-1640.
- BARGER, I.A. (1993) Influence of sex and reproductive status on the susceptibility of ruminants to nematode parasitism. *International Journal for Parasitology* **23**, 463-469.
- BARRY, T.N., ALLSOP, T.F. AND REDEKOPP, C. (1986a) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 5. Effects on the endocrine system on adipose tissue metabolism. *British Journal of Nutrition* **56**, 607-614.
- BARRY, T.N. AND DUNCAN, S.J. (1984) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 1. Voluntary intake. *British Journal of Nutrition* **51**, 485-491.
- BARRY, T.N. AND MANLEY, T.R. (1984) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 2. Quantitative digestion of carbohydrates and proteins. *British Journal of Nutrition* **51**, 493-504.
- BARRY, T.N. AND MANLEY, T.R. (1986) Interrelationships between the concentrations of total condensed tannin, free condensed tannin and lignin in *Lotus sp.* and their possible consequences in ruminant nutrition. *Journal of the Science of Food and Agriculture* **37**, 248-254.
- BARRY, T.N., MANLEY, T.R. AND DUNCAN, S.J. (1986b) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary tannin concentration. *British Journal of Nutrition* **55**, 123-137.
- BARRY, T.N. AND McNABB, W.C. (1999) The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition* **81**, 263-272.



- BASS, J.J., SPENCER, G.S.G. AND HODGKINSON, S.G. (1992) Nutritional control of the growth hormone axis. In *The Control of Fat and Lean Deposition*, pp. 175-196 (Eds P.J. Buttery, K.N. Boorman and D.B. Lindsay) Butterworth-Heinemann Ltd., Oxford, UK
- BAWDEN, R.J. (1969) The establishment and survival of *Oesophagomum columbianum* in male and female sheep given high and low protein diets. *Australian Journal of Agricultural Research* 20, 1151-1159.
- BEEVER, D.E. AND SIDDONS, R.C. (1986) Digestion and metabolism in the grazing ruminant. In *Control of Digestion and Metabolism in Ruminants*, pp 479-497 (Eds L.P. Milligan, W.L. Grovum and A. Dobson) Prentice-Hall, New Jersey.
- BENNET-JENKINS, E. AND BRYANT, C. (1996) Novel sources of anthelmintics. *International Journal for Parasitology* 26, 937-947.
- BERNAYS, E.A., DRIVER, G.C. AND BILGENER, M. (1989) Herbivores and plant tannins. *Advances in Ecological Research* 19, 263-302.
- BIRD, S.H. AND LENG, R.A. (1985) Productivity responses to eliminating protozoa from the rumen of sheep. In: *Reviews in Rural Science 6. Biotechnology and Recombinant DNA Technology in the Animal Production Industries*, pp. 109-117 (Eds R.A. Leng, J.S.F. Barker, D.B. Adams and K.J. Hutchinson).
- BLYTT, H.J., GUSCAR, T.K. AND BUTLER, L.G. (1988) Antinutritional effects and ecological significance of dietary condensed tannins may not be due to binding and inhibiting digestive enzymes. *Journal of Chemical Ecology*, 14, 1455-1465.
- BOOKER, J.D., O'DONOVAN, L.A., SKENE, I., CLARKE, K., BLACKALL, L. AND MUSLERA, P. (1994) *Streptococcus caprinus* sp.nov., a tannin-tolerant ruminal bacterium from feral goats. *Letters in Applied Microbiology* 18, 313-318.
- BOWN, M.D., POPPI, D.P. AND SYKES, A.R. (1989) The effects of a concurrent infection of *Trichostrongylus colubriformis* and *Ostertagia circumcincta* on calcium, phosphorus and magnesium transactions along the digestive tract of lambs. *Journal of Comparative Pathology* 101, 11-20.
- BOWN, M.D., POPPI, D.P. AND SYKES, A.R. (1991a) Nitrogen transactions along the digestive tract of lambs concurrently infected with *Trichostrongylus colubriformis* and *Ostertagia circumcincta*. *British Journal of Nutrition* 66, 237-249.
- BOWN, M.D., POPPI, D.P. AND SYKES, A.R. (1991b) The effect of post-ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus colubriformis* infection and body composition in lambs. *Australian Journal of Agricultural Research* 42, 253-267.



- BRAVO, L., ABIA, R., EASTWOOD, M.A. AND SAURA-CALIXTO, F. (1994) Degradation of polyphenols (catchin and tannic acid) on the rat intestinal tract. Effect on colonic fermentation and faecal output. *British Journal of Nutrition* 71, 933-949.
- BROPHY, P.M. AND BARRETT, J. (1990) Glutathione transferase in helminths. *Parasitology* 100, 345-349.
- BROPHY, P.M., PAPADOULOS, A., TOURAKI, M., COLES, B., KÖRTING, W. AND BARRETT, J. (1989) Purification of cytosolic glutathione transferases from *Schistocephalus solidus* (plerocercoid): interaction with anthelmintics and products of lipid peroxidation. *Molecular and Biochemical Parasitology* 36, 187-196.
- BURTIS, C.A AND ASHWOOD, E.R. (1994) *Tietz Textbook of Clinical Chemistry*. 2nd Edition. W.B. Saunders Company, Harcourt Brace & Co., Philadelphia.
- BUTLER, L.G. (1989) Effects of condensed tannin on animal nutrition. In: chemistry and Significance of Condensed Tannins. Editors R.W. HEMINGWAY AND J.J. KARCHESY. Plenum Press, New York, 391-401.
- BUTLER, L.G., ROGLER, J.C., MEHANSHO, H. AND CARLSON, D.M. (1986) Dietary effects of tannins. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structural Activity Relationships*, pp. 141-157 (Eds. V. Cody, E. Middleton, and J.B. Harbone) Alan R. Liss, Inc. New York.
- CABARET, J., GASNIER, N. AND JACQUIET, P. (1998) Faecal egg counts are representative of digestive-tract strongyle worm burdens in sheep and goats. *Parasite* 5, 137-142.
- CHANDEL, Y.S. AND METHA, P.K. (1990) nematocidal properties of leaf extract of wild sage (*Lantana camara*). *Indian Journal of Agricultural Science* 60, 781.
- CHANDRA, R.K. (1993) Nutrition and the immune system. *Proceedings of the Nutrition Society* 52, 77-84.
- CHEEMA, K.J. AND SCOFIELD, A.M. (1982) Intestinal absorption of glucose in rats after continuous challenge with larvae of *Nippostrongylus brasiliensis*. *Journal of Helminthology* 58, 263-266.
- CHIQUETTE, J., CHENG, K-J., COSTERTON, J.W. AND MULLIGAN, L.P. (1988) Effect of tannins on the digestibility of two isosynthetic strains of birdsfoot trefoil (*Lotus corniculatus* L.) using *in vitro* and *in sacco* techniques. *Canadian Journal of Animal Science* 68, 751-760.
- CHRISTIE, M. AND JACKSON, F. (1982) Specific identification of strongyle eggs in small samples of sheep faeces. *Research in Veterinary Science* 32, 113-117.
- CLARKE, R.T.J. AND REID, C.S.W. (1972) Foamy bloat of cattle: A review. *Journal of Dairy Science* 57, 753-785.



- COOP, R.L. (1998) Nutrition and parasite interaction - a review. In: *Moredun Research Institute Annual Report 1997*, pp. 43-49.
- COOP, R.L. AND ANGUS, K.W. (1975) The effect of continuous doses of *Trichostrongylus colubriformis* larvae on the intestinal mucosa of sheep and on liver vitamin A concentration. *Parasitology* 70, 1-9.
- COOP, R. AND FIELD, A.C. (1983) Effect of phosphorus intake on growth rate, food intake and quality of the skeleton of growing lambs infected with the intestinal nematode *Trichostrongylus vitrinus*. *Research in Veterinary Science* 35, 175-181.
- COOP, R.L. AND HOLMES, P.H. (1996) Nutrition and parasite interaction. *International Journal for Parasitology* 26, 951-962.
- COUSINS, B.W., TANKSLEY, T.D., KNABE, D.A. AND ZEBROWSKA, T. (1981) Nutrient digestibility and performance of pigs fed sorghums varying in tannin concentration. *Journal of Animal Science* 53, 1524-1537.
- COX, F.E.G. (1993) Immunology. In: *Modern Parasitology. A textbook of parasitology* pp 193-218 (Ed. F.E.G. Cox) 2nd Edition, Blackwell Scientific Publications Inc., Oxford.
- CROFTON, H.D. (1971) A quantitative approach to parasitism. *Parasitology* 62, 179-193.
- CROMPTON, D.W.T., WALTERS, D.E. AND ARNOLD, S. (1981) Changes in the food intake and body weight of protein-malnourished rats infected with *Nippostrongylus brasiliensis* (Nematoda). *Parasitology* 82, 23-38.
- CUMMINS, A.G., DUNCOMBRE, V.M., BOLIN, T.D., DAVIS, A.E. AND KELLY, J.D. (1978) Suppression of rejection of *Nippostrongylus brasiliensis* in iron and protein deficient rats: effect of synthetic lymphocyte transfer. *Gut* 19, 823-826.
- CUMMINS, A.G., DUNCOMBRE, V.M., BOLIN, T.D., DAVIS, A.E. AND YONG, J. (1987) The response of the small intestine of the protein-deficient rat to infection with *Nippostrongylus brasiliensis*. *International Journal for Parasitology* 17, 1445-1450.
- DATTA, F.U., NOLAN, J.V., ROWE, J.B. AND GRAY, G.D. (1998) Protein supplementation improves the performance of parasitised sheep fed a straw-based diet *International Journal for Parasitology* 28, 1269-1278.
- DAWKINS, H.J.S., WINDON, R.G. AND EAGLESON, G.K. (1989) Eosinophil responses in sheep selected for high and low responses to *Trichostrongylus colubriformis*. *International Journal for Parasitology* 19, 199-205.



- DAWKINS, H.J.S., WINDON, R.G., OUTERIDGE, P.M. AND DINEEN, J.K. (1988) Cellular and humoral responses of sheep with different levels of resistance to *Trichostrongylus colubriformis*. *International Journal for Parasitology* 18, 531-537.
- DAWSON, J.M., BUTTERY, P.J., JENKINS, D., WOOD, C.D. AND GILL, M. (1999) Effect of dietary quebracho tannin on nutrient utilisation and tissue metabolism in sheep and rats. *Journal of the Science of Food and Agriculture* 79, 1-8.
- DAWSON, J.M., GREATHEAD, H.M.R., CRAIGON, J., HACHEY, D.L., REEDS, P.J., PELL, J.M. AND BUTTERY, P.J. (1998) The interaction between nutritional status and growth hormone in young cattle: differential responsiveness of fat and protein metabolism. *British Journal of Nutrition* 79, 275-286.
- DEGEN, A.A., BLANKE, A., BECKER, K., KAM, M., BENJAMIN, R.W. AND MAKKAR, H.P.S (1997) The nutritive value of *Acacia saligna* and *Acacia salicina* for goats and sheep *Animal Science* 64, 253-259.
- DESHPANDE, S.S. AND SALUNKHE, D.F. (1982) Interactions of tannic acid and catechin with legume starches. *Journal of Food Science* 47, 2080-2081.
- D'MELLO, J.P.F. (1992) Chemical constraints to the use of tropical legumes in animal nutrition. *Animal Feed Science Technology* 38, 237-261.
- DOBSON, C. AND BAWDEN, R.J. (1974) Studies on the immunity of sheep to *Oesophagostomum columbianum*: effects of low-protein diet on the resistance to infection and cellular reactions in the gut. *Parasitology* 69, 239-255.
- DOLLAHITE, J.W., PIGEON, R.F. AND CAMP, B.J. (1962) Oral toxicity and polyhydroxphenol moiety of tannin isolated from *Quertus havardi* in the rabbit. *American Journal of Veterinary Science* 23, 1268-1273.
- DONALDSON, J., VAN HOUTERT, M.F.J. AND SYKES, A.R. (1997) The effect of protein supply on the periparturient status of the mature ewe. *Proceedings of the New Zealand Society of Animal Production* 57, 186-189.
- DOUCH, P.G.C. (1990) The sheep's immune response to nematode parasites and prospects for its exploitation. *Proceedings of the New Zealand Society of Animal Production* 50, 229-236.
- DOUCH, P.G.C., HARRISON, G.B.L., ELLIOTT, D.C. AND BUCHANAN, L.L. (1986) Relationship of gastrointestinal histology and mucus antiparasite activity with the development of resistance to trichostrongyle infections in sheep. *Veterinary Parasitology* 20, 315-331.
- DOUGLAS, G.B., WANG, Y., WAGHORN, G.C., BARRY, T.N., PURCHAS, R.W., FOOTE, A.G. AND WILSON, G.F. (1995) Liveweight gain and wool production of sheep grazing *Lotus corniculatus* and lucerne (*Medicago saliva*). *New Zealand Journal of Agricultural Research* 38, 95-104.



- DYNES, R.A., ANKERSMIT, A.E.L., POPPI, D.P., BARRELL, G.K. AND SYKES, A.R. (1990) Studies on the physiological basis of appetite depression in nematode infection in sheep. *Proceedings of the New Zealand Society of Animal Production* 50, 249-253.
- DYNES, R.A., POPPI, D.P., BARRELL, G.K. AND SYKES, A.R. (1998) Elevation of feed intake in parasite infected lambs by central administration of a cholecystokinin receptor antagonist. *British Journal of Nutrition* 79, 47-54.
- EADY, S.J., WOOLASTON, R.R., MORTIMER, S.I., LEWER, R.P., READSMA, H.W., SWAN, A.A. AND PONZONI, R.W. (1996) Resistance to nematode parasites in Merino sheep: sources of genetic variation. *Australian Journal of Agricultural Research* 47, 895-915.
- EGAN, A.R. AND ULYATT, M.J. (1980) Quantitative digestion of fresh herbage by sheep. VI Utilisation of nitrogen in five herbages. *The Journal of Agricultural Science, Cambridge* 94, 47-56.
- ELKIN, R.G., ROGLER, J.C. AND SULLIVAN, T.W. (1990) Comparative effects of dietary tannins in ducks, chicks and rats. *Poultry Science* 69, 1685-1693.
- FAHEY, G.C. AND JUNG, H.J.G. (1989) In *Toxicants of Plant Origin, vol. IV, Phenolics*, pp 123-190 (Ed. P.R. Cheeke) Boca Raton, Florida: CRC Press
- FEATHERSTON, W.R. AND ROGLER, J.C. (1975) Influence of tannins on the utilisation of sorghum grain by rats and chicks. *Nutrition Reports international* 11, 491-497.
- FERGUSON, E.G.W., MITCHELL, G.B.B. AND MACPHERSON, A. (1989) Cobalt deficiency and *Ostertagia circumcincta* infection in lambs. *Veterinary Record* 124, 20.
- FOO, L.Y., HRSTICH, L. AND VILAIN, C. (1985) Phylloflavan, a characteristic constituent of *Phyllocladus* species. *Phytochemistry* 24, 1495-1498.
- FOO, L.Y., LU, Y., MCNABB, W.C., WAGHORN, G.C. AND ULYATT, M.J. (1997) Proanthocyanidins from *Lotus pedunculatus*. *Phytochemistry* 45, 1689-1696.
- FOO, L.Y., NEWMAN, R., WAGHORN, G.C., MCNABB, W.C. AND ULYATT, M.J. (1996) The proanthocyanidins of *Lotus corniculatus*. *Phytochemistry* 41, 617-624.
- FRANDSEN, J.C. (1985) Effects of restriction in dietary protein and vitamin A on the responses of rats to infections by *Nippostrongylus brasiliensis* (Nematoda) and *N. brasiliensis* and *Eimeria nieschulzi* (Coccidia). *International Journal for Parasitology* 15, 523-528.
- GIBSON, T.E. (1963) The influence of nutrition on the relationships between gastrointestinal parasites and their hosts. *Proceedings of the Nutrition Society* 22, 15-20.



- GILL, H.S. AND LEJAMBRE, L.F. (1996) Preface- Novel approaches to the control of helminth parasites of livestock. *International Journal for Parasitology* 26, 797-798.
- GRIFFITHS, D.W. AND MOSELEY, G. (1980) The effect of diets containing field beans of high or low polyphenolic content on the activity of digestive enzymes in the intestines of rats. *Journal of the Science of Food and Agriculture* 31, 255-259.
- HAGERMAN, A.E. (1989) Chemistry of tannin-protein complexation. In: *Chemistry and significance of condensed tannins*, pp. 323-333 (Eds R.W. Hemingway and J.J. Karchasy). Plenum Press, New York.
- HAGERMAN, A.E. AND BUTLER, L.G. (1981) The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry* 256, 4494-4497.
- HARRISON, G.B.L., PULFORD, H.D., GATEHOUSE, T.K., SHAW, R.J., PFEFFER, A. AND SHOEMAKER, C.B. (1999) Studies on the role of mucus and mucosal hypersensitivity reactions during rejection of *Trichostrongylus colubriformis* from the intestine of immune sheep using an experimental challenge model. *International Journal for Parasitology* 29, 459-468.
- HENIS, Y., TAGARI, H. AND VOLCANI, R. (1964) Effect of water extracts of carob pods, tannic acid and their derivatives on the morphology and growth of microorganisms. *Applied Microbiology* 12, 204-209.
- HORBURY, S.R., MERCER, J.G. AND CHAPPELL, L.H. (1995) Anorexia induced by the parasitic nematode. *Nippostrongylus brasiliensis*: effects on NPY and CRF gene expression in the rat hypothalamus. *Journal of Neuroendocrinology* 7, 867-873.
- HORIGOME, T., KUMAR, R. AND OKAMOTO, K. (1988) Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes *in vitro* and in the intestine of rats. *British Journal of Nutrition* 60, 275-285.
- VAN HOUTERT, M.F.H., BARGER, I.A. AND STEEL, J.W. (1995a) Dietary protein for young grazing sheep: interactions with gastrointestinal parasitism. *Veterinary Parasitology* 60, 283-295.
- VAN HOUTERT, M.F.J., BARGER, I.A. AND STEEL, J.W. (1996) Supplementary feeding and gastrointestinal nematode parasitism in young grazing sheep. *Proceedings of the New Zealand Society of Animal Production* 56, 94-98.
- VAN HOUTERT, M.F.J., BARGER, I.A., STEEL, J.W., WINDON, R.G. AND EMERY, D.L. (1995b) Effects of dietary protein intake on responses of young sheep to infection with *Trichostrongylus colubriformis*. *Veterinary Parasitology* 56, 163-180.
- HUME, I.D., MUIR, R.J. AND SOMERS, M. (1970) Synthesis of microbial protein in the rumen. I Influence of the level of nitrogen intake. *Australian Journal of Agricultural Research* 21, 283-296.



- HUNTLEY, J.F., NEWLANDS, G. AND MILLER, H.R.P. (1984) The isolation and characterisation of globule leukocytes; their derivation from mucosal mast cells in parasitised sheep. *Parasite Immunology* 6, 371-390.
- JACKSON, F., ANGUS, K.W. AND COOP, R.L. (1983) Development of morphological changes in the small intestine of lambs continuously infected with *Trichostrongylus vitrinus*. *Research in Veterinary Science* 34, 316-323.
- JAMBUNATHAN, R. AND MERTZ, E.T. (1973) Relationship between tannin levels, rat growth, and distribution of proteins in sorghum. *Journal of Agriculture and Food Chemistry* 21, 692-696.
- JANSMAN, A.J.M. (1993) Tannins in feedstuffs for simple-stomached animals. *Nutrition Research Reviews* 6, 209-236.
- JARRETT, E.E., JARRETT, W.F.H. AND URQUHART, G.M. (1968) Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptible and immune hosts. *Nippostrongylus brasiliensis* in the rat. *Parasitology* 58, 625-639.
- JONES, D.A. (1993) The eosinophil. *Journal of Comparative Pathology* 108, 317-335.
- JONES, G.A., MCALLISTER, T.A., MUIR, A.D. AND CHENG, K.-J. (1994) Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of rumen bacteria. *Applied and Environmental Microbiology* 60, 1374-1378.
- JONES, W.T. (1976) The condensed tannin of pasture legume species. *Phytochemistry* 15, 1407-1409.
- JONES, W.T., ANDERSON, L.B. AND ROSS, M.D. (1973) Bloat in cattle. XXXIX Detection of protein precipitants (flavolans) in legumes. *New Zealand Journal of Agricultural Research* 16, 441-446.
- JONES, W.T., LYTTLETON, J.W. AND CLARKE, R.T.J. (1970) Bloat in cattle. 33. The soluble proteins of legume forages in New Zealand and their relationship to bloat. *New Zealand Journal of Agricultural Research* 13, 149-157.
- JONES, W.T., LYTTLETON, J.W. AND MANGAN, J.L. (1978) Interactions between fraction 1 leaf protein and other surfactants involved in the bloat syndrome. *New Zealand Journal of Agricultural Research* 21, 401-407.
- JONES, W.T. AND MANGAN, J.L. (1977) Complexes of the condensed tannin of sainfoin (*Onobrychis vicifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *Journal of the Science of Food and Agriculture* 28, 126-136.

- KAMBARA, T., MCFARLANE, R.G., ABELL, T.J., MCANULTY, R.W. AND SYKES, A.R. (1993) The effect of age and dietary protein on immunity and resistance in lambs vaccinated with *Trichostrongylus colubriformis*. *International Journal for Parasitology* 23, 471-476.
- KAWALEK, J.C., REW, R.S. AND HAEVNER, J. (1984) Glutathione S-transferase, a possible drug metabolising enzyme in *Haemonchus contortus*: comparative activity of a cambendazole resistant and a susceptible strain. *International Journal for Parasitology* 14, 173-177.
- KENDALL, W.A. (1966) Factors affecting foams with forage legumes *Crop Science* 6, 487-489.
- KEYMER, A., CROMPTON, D.W.T. AND SAHAKIAN, B.J. (1983) Parasite-induced learned taste aversion involving *Nippostrongylus* in rats. *Parasitology* 86, 455-460.
- KIMAMBO, A.E., MACRAE, J.C., WALKER, A., WATT, C.F. AND COOP, R.L. (1988) Effect of prolonged subclinical infection with *Trichostrongylus colubriformis* on the performance and nitrogen metabolism of growing lambs. *Veterinary Parasitology* 28, 191-203.
- KOTB, A.R. AND LUCKEY, T.D. (1973) Markers in nutrition. *Nutrition Abstracts and Reviews* 42, 813-845.
- KUMAR, R. AND VIATHIYANATHAN, S. (1990) Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. *Animal Feed Science and Technology* 30, 21-38.
- KYRIAZAKIS, I., ANDERSON, D.H., COOP, R.L. AND JACKSON, F. (1996a) The pathophysiology and development of immunity during long-term subclinical infection with *Trichostrongylus colubriformis* of sheep receiving different nutritional treatments. *Veterinary Parasitology* 65, 41-54.
- KYRIAZAKIS, I., ANDERSON, D.H., OLDHAM, J.D., COOP, R.L. AND JACKSON, F. (1996b) Long-term subclinical infection with *Trichostrongylus colubriformis*: Effects on food intake, diet selection and performance of growing lambs. *Veterinary Parasitology* 61, 297-313.
- KYRIAZAKIS, I., OLDHAM, J.D., COOP, R.L. AND JACKSON, F. (1994) The effect of subclinical intestinal nematode infection on the diet selection of growing sheep. *British Journal of Nutrition* 72, 665-677.
- KYRIAZAKIS, I., TOLKAMP, B.J. AND HUTCHINGS, M.R. (1998) Towards a functional explanation for the occurrence of anorexia during parasitic infections. *Animal Behaviour* 56, 265-274.



- LEE, J., HARRIS, P.M., SINCLAIR, B.R. AND TRELOAR, B.P. (1995) Sulfur amino acid metabolism and protein synthesis in young sheep fed ryegrass pasture and two lotus cultivars containing condensed tannin. *Australian Journal of Agricultural Research* 46, 1587-1600.
- VAN LEEUWEN, P., JANSMAN, A.J.M., WIEBENGA, J., KONINKX, J.F.J.G. AND MOUWEN, J.M.V.M. (1995) Dietary effects of faba-bean (*Vicia faba* L.) tannins on the morphology and function of the small-intestinal mucosa of weaned pigs. *British Journal of Nutrition* 73, 31-39.
- VAN LEEUWEN, P., VELDMAN, A., BOISEN, S., DEURING, K., VAN KEMPEN, G.J.M., DERKSEN, G.B., VERSTEGEN, M.W.A. AND SCHAAFSMA, G. (1996) Apparent ileal dry matter and crude protein digestibility of rations fed to pigs and determined with the use of chromic oxide (Cr<sub>2</sub>O<sub>3</sub>) and acid-insoluble ash as digestive markers. *British Journal of Nutrition* 76, 551-562.
- LINDSAY, J.H., HOGAN, A.E. AND DONNELLY, J.B. (1980) The digestion of protein from forage diets in the small intestine of the sheep. *Australian Journal of Agricultural Research* 31, 589-600.
- LLOYD, S. AND SOULSBY, E.J.L. (1987): Immunology of gastrointestinal nematodes of ruminants. In: Immune responses in parasite infections: immunology, immunopathology and immunoprophylaxis. Vol. 1: Nematodes, pp 1-42. (Ed. E.J.L. Soulsby) CRC Press Inc., Boca Raton, Florida.
- LORIMER, S.D., PERRY, N.B., FOSTER, L.M. AND BURGESS, E.L. (1996) A nematode larval motility inhibition assay for screening plant extracts and natural products. *Journal of Agricultural and Food Chemistry* 44, 2842-2845.
- LUNN, P.G., NORTHROP, C.A. AND WAINWRIGHT, M. (1988) Hypoalbuminemia in energy-malnourished rats infected with *Nippostrongylus brasiliensis* (Nematoda). *The Journal of Nutrition* 118, 121-127.
- MAHMOOD, S. AND SMITHARD, R. (1993) A comparison of effects of body weight and feed intake on digestion in broiler cockerels with effects of tannins. *British Journal of Nutrition* 70, 701-709.
- MAKKAR, H.P.S., BECKER, K., ADEL, H. AND SZEGLETTI, C. (1995a) Degradation of condensed tannins by rumen microbes exposed to quebracho tannin (QT) in Rumen simulation technique (RUSITEC) and effects of QT on fermentative processes in the RUSITEC. *Journal of the Science of Food and Agriculture* 69, 495-500.
- MAKKAR, H.P.S., BLUMMEL, M. AND BECKER, K. (1995b) *In vitro* effects of and interactions between tannins and saponins and fate of tannins in the rumen. *Journal of the Science of Food and Agriculture* 69, 481-493.
- MAKKAR, H.P.S., SINGH, B. AND DAWRA, R.K. (1987) Tannin-nutrient interactions- A review. *International Journal of Animal Science* 2, 127-140.



- MAKKAR, H.P.S., SINGH, B. AND DAWRA, R.K. (1988) Effect of tannin-rich leaves of oak (*Quercus incana*) on various microbial enzyme activities of the bovine rumen. *British Journal of Nutrition* 60, 287-296.
- MALLOY, M.H., RASSIN, D.K. AND GAULL, G.E. (1981) A method for measurement of free and bound plasma cyst(e)ine. *Analytical Biochemistry* 113, 407-415.
- MANGAN, J.L. (1988) Nutritional effects of tannins in animal feeds. *Nutrition Research Reviews* 1, 209-231.
- MARTIN, J.S. AND MARTIN, M.M. (1983) Tannin assays in ecological studies. Precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid, quebracho and oak foliage extracts. *Journal of Chemical Ecology* 9, 285-294.
- MARTIN, M.M., ROCKHOLM, D.C. AND MARTIN, J.S. (1985) Effects of surfactants, pH and certain cations on precipitation of proteins by tannins. *Journal of Chemical Ecology* 11, 485-494.
- MCCLURE, S.J., EMERY, D.L., WAGLAND, B.M. AND JONES, W.O. (1992) A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *International Journal for Parasitology* 22, 227-234.
- MCDONALD, P., EDWARDS, R.A., GREENHALGH, J.F.D. AND MORGAN, C.A. (1995) *Animal Nutrition* 5<sup>th</sup> Edition. Longman Scientific and Technical, Harlow.
- M McNABB, W.C., WAGHORN, G.C., BARRY, T.N. AND SHELTON, I.D. (1993) The effect of condensed tannin in *Lotus pedunculatus* on the digestion and metabolism of methionine, cystine and inorganic sulphur in sheep. *British Journal of Nutrition* 70, 647-661.
- MEHANSHO, H., BUTLER, L.G. AND CARLSON, D.M. (1987) Dietary tannins and salivary proline-rich proteins: interactions, induction, and defence mechanisms. *Annual Review of Nutrition* 7, 423-440.
- MILLER, H.R.P. (1984) The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Veterinary Immunology and Immunopathology* 6, 167-259.
- MILLER, H.R.P. (1987) Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. *Parasitology* 94, s77-s100.
- MILLER, T.A. (1979) Hookworm infection in man. *Advances in Parasitology* 17, 315-384.
- MILLER, H.R.P. AND HUNTLEY, J.F. (1982) Protection against nematodes by intestinal mucus. In: *Mucus in Health and Disease*, pp. 243-261 (Eds E.N. Chantler, J.B. Elder and M. Elstein) Plenum Press, New York.



- MILLER, H.R.P. AND NAWA, Y. (1979) Immune regulation of intestinal goblet cell differentiation. Specific induction of non-specific protection against helminths. *Nouvelle Revue Francaise D Hematologie* 21, 31-35.
- MILLER, H.R.P., HUNTLEY, J.F. AND WALLACE, G.R. (1981) Immune exclusion and mucus trapping during the rapid expulsion of *Nippostrongylus brasiliensis* from primed rats. *Immunology* 44, 73-76.
- MILLER, H.R.P., JACKSON, F., NEWLANDS, G.F.J. AND HUNTLEY, J.F. (1985) Rapid expulsion of gastrointestinal nematodes in the sheep: a role for immediate hypersensitivity reactions in the mucosa. In: *Immunology of the Sheep* pp. 460-479 (Eds B. Morris and M. Miyasaka) Editions Roche, Basel.
- MILLER, S.M., PRITCHARD, D.A., EADY, S.J. AND MARTIN, P.R. (1997) Polyethelene glycol is more effective than surfactants to enhance digestion and production in sheep fed mulga (*Acacia aneura*) under pen and paddock conditions. *Australian Journal of Agricultural Research* 48, 1121-1127.
- MIN, B.R., BARRY, T.N., WILSON, P.R AND KEMP, P.D. (1997) The effects of grazing chicory (*Cichorium intybus*) and birdsfoot trefoil (*Lotus corniculatus*) on venison and velvet production by young red and hybrid deer. *New Zealand Journal of Agricultural Research* 40, 335-347.
- MITARU, B.N., REICHERT, R.D. AND BLAIR, R. (1984) The binding of dietary protein by sorghum tannins in the digestive tract of pigs. *The Journal of Nutrition* 114, 1787-1796.
- MITJAVILA, S., LACOMBE, C., CARRERA, G. AND DERACHE, R. (1977) Tannic acid and oxidised tannin acid on the functional state of rat intestinal epithelium. *The Journal of Nutrition* 107, 2113-2121.
- MOLE, S. (1989) Polyphenolics and the nutritional ecology of herbivores. In: *Toxicants of plant origin. IV. Phenolics*, pp. 191-223 (Ed. P.R. Cheeke) CRC Press, Boca Raton, FL.
- MOLE, S., ROGLER, J.C. AND BUTLER, L.G. (1993) Growth reduction by dietary tannins: Different effects due to different tannins. *Biochemical Systematics and Ecology* 21, 667-677.
- MOLE, S. AND WATERMAN, P.G. (1987) Tannic acid and proteolytic enzyme inhibition or substrate deprivation? *Phytochemistry* 26, 99-102.
- MUELLER-HARVEY, I. AND HARTLEY, R.D. (1987) Characteristics of phenolic compounds, including flavanoids and tannins, of ten Ethiopian browse species by high performance liquid chromatography. *Journal of the Science of Food and Agriculture* 39, 1-14.
- MUELLER-HARVEY, I. AND MCALLAN, A.B. (1992) Tannins. Their biochemistry and nutritional properties. *Advances in Plant Cell Biochemistry and Biotechnology* 1, 151-217.



- MURDIATI, T.B., MCSWEENEY, C.S. AND LOWRY, J.B. (1992) Metabolism in sheep of gallic acid, tannic acid and hydrolysable tannin from *Terminalia oblongata*. *Australian Journal of Agricultural Research* 43, 1307-1319.
- NAWA, Y. (1979) Increased permeability of gut mucosa in rats infected with *Nippostrongylus brasiliensis*. *International Journal for Parasitology* 9, 251-255.
- NATIONAL RESEARCH COUNCIL (1995) *Nutritent Requirements of Laboratory Animals* 4<sup>th</sup> Revised Edition. National Academy Press, Washington D.C.
- NELSON, K.E., PELL, A.N., SCHOFIELD, P. AND ZINDER, S. (1995) Isolation and characterisation of an anaerobic ruminal bacterium capable of degrading hydrolysable tannins. *Applied and Environmental Microbiology* 61, 3293-3298.
- NELSON, K.E., THONNEY, M.L., WOOLASTON, T.K., ZINDER, S. AND PELL, A.N. (1998) Phenotypic and phlogenetic characterization of ruminal tannin-tolerant bacteria. *Applied and Environmental Microbiology* 64, 3824-3830.
- NDAMUKONG, K.J.N. AND NGONE, M.M. (1996) Development and survival of *Haemonchus contortus* and *Trichostrongylus* spp. on pasture in Cameroon. *Tropical Animal Health and Production* 28, 193-197.
- NIEZEN, J.H., CHARLESTON, W.A.G., HODGSON, J., MACKAY, A.D. AND LEATHWICK, D.M. (1996) Controlling internal parasites in grazing ruminants without recourse to anthelmintics: Approaches, Experiences and Prospects. *International Journal for Parasitology* 26, 983-992.
- NIEZEN, J.H., ROBERTSON, H.A., WAGHORN, G.C. AND CHARLESTON, W.A.G. (1998a) Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. *Veterinary Parasitology* 80, 15-27.
- NIEZEN, J.H., WAGHORN, T.S., CHARLESTON, W.A.G. (1998b) Establishment and fecundity of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in lambs fed lotus (*Lotus pedunculatus*) or perennial ryegrass (*Lolium perenne*). *Veterinary Parasitology* 78, 13-21.
- NIEZEN, J.H., WAGHORN, T.S., CHARLESTON, W.A.G. AND WAGHORN, G.C. (1995) Growth and gastrointestinal nematode parasitism in lambs grazing lucerne (*Medicago saliva*) or sulla (*Hedysarum coronarium*) which contains condensed tannins. *The Journal of Agricultural Science, Cambridge* 125, 281-289.
- NIEZEN, J.H., WAGHORN, T.S., WAGHORN, G.C. AND CHARLESTON, W.A.G. (1993) Internal parasites and lamb production - a role for plants containing condensed tannins? *Proceedings of the New Zealand Society of Animal Production* 53, 235-238.



- ODENYO, A.A., OSUJI, P.O. (1998) Tannin-tolerant ruminal bacteria from East African ruminants. *Canadian Journal of Microbiology*, **44**, 905-909.
- OH, N.I. AND HOFF, J.E. (1986) Effect of condensed grape tannins on the *in vitro* activity of digestive proteases and activation of their zymogens *Journal of Food Science* **51**, 577-580.
- ORTIZ, L.T., ALZUETA, C., TREVINO, J. AND CASTANO, M. (1994) Effects of faba bean tannins on the growth and histological structure of the intestinal tract and liver of chicks and rats. *British Poultry Science* **35**, 743-754.
- OWENS, F.N AND GOETSCH, A.L. (1986) Digesta passage and microbial protein synthesis. In: *Control of Digestion and Metabolism in Ruminants* Editors L.P. Milligan, W.L. Grovum and A. Dobson pp. 196-223 Prentice-Hill, New Jersey, USA.
- PARKINS, J.J. AND HOLMES, P.H. (1989) Effects of gastrointestinal helminth parasites on ruminant nutrition. *Nutrition Research Reviews* **2**, 227-246.
- PARRINDER, R., ATKINSON, J.L., DAWSON, J.M., BUTTERY, P.J. AND GILL, M. (1993a) Preliminary studies on the fate of the flavanol glycoside rutin in the forestomachs of sheep. In: *Animal Production in Developing Countries* (Occasional Publication No. 16) pp. 200-201 (abstract) (Eds M. Gill, E. Owen, G.E. Pollott and T.J.L. Lawrence) British Society of Animal Production, Edinburgh, UK.
- PARRINDER, R., BUTTERY, P.J., DAWSON, J.M., GILL, AND WOOD, C.D. (1993b) The fate of low molecular weight polyphenolics in the rumen of sheep. *Animal Production* **56**, 465 (abstract)
- POPPI, D.P., MACRAE, J.C., BREWER, A. AND COOP, R.L. (1986) Nitrogen transactions in the digestive tract of lambs exposed to the intestinal parasite *Trichostrongylus colubriformis*. *British Journal of Nutrition* **55**, 593-602
- POPPI, D.P., MACRAE, J.C., BREWER, A.C., DEWEY, P.J.S. AND WALKER, A. (1985) Calcium and phosphorus absorption in lambs exposed to *Trichostrongylus colubriformis*. *Journal of Comparative Pathology* **95**, 453-464.
- POPPI, D.P., SYKES, A.R. AND DYNES, R.A. (1990) The effect of endoparasitism on host nutrition - the implications for nutrient manipulation. *Proceedings of the New Zealand Society of Animal Production* **50**, 237-243.
- PRICHARD, D.A., MARTIN, P.R. AND O'ROURKE, P.K. (1992) The role of condensed tannins in the nutritional value of mulga (*Acacia aneura*) for sheep. *Australian Journal of Agricultural Research* **43**, 1739-1746.
- PRICHARD, D.A., STOCKS, D.C., O'SULLIVAN, B.M., MARTIN, P.R., HURWOOD, I.S. AND O'ROURKE, P.K. (1988) The effect of polyethylene glycol (PEG) on wool growth and liveweight of sheep consuming a mulga (*Acacia aneura*) diet. *Proceedings of the Australian Society of Animal Production* **17**, 290-293.



- PURCHAS, R.W. AND KEOGH, R.G. (1984) Fatness of lambs grazed on 'Grasslands Maku' lotus and 'Grasslands Huia' white clover. *Proceedings of the New Zealand Journal of Animal Production* 44, 219-221.
- REED, J.D., SOLLER, H. AND WOODWARD, A. (1990) Fodder tree and straw diets for sheep: intake, growth, digestibility and the effects of phenolics on nitrogen utilisation. *Animal Feed Science and Technology* 30, 39-50.
- RITTNER, U. AND REED, D. (1992) Phenolics and *in vitro* degradability of protein and fibre in West African browse. *Journal of the Science of Food and Agriculture* 58, 21-28.
- ROBBINS, C.T., HAGERMAN, A.E., AUSTIN, P.J., MCARTHUR, C. AND HANLEY, T.A. (1991) Variation in mammalian physiological responses to a condensed tannin and its ecological implications. *Journal of Mammalogy* 72, 480-486.
- ROBERTSON, H.A., NIEZEN, J.H., WAGHORN, G.C., CHARLESTON, W.A.G. AND JINLONG, M. (1995) The effect of six herbages on liveweight gain, wool growth and faecal egg count of parasitised ewe lambs. *Proceedings of the New Zealand Society of Animal Production* 55, 199-201.
- ROTHWELL, T.L.W. (1989) Immune expulsion of parasitic nematodes from the alimentary tract. *International Journal for Parasitology* 19, 139-168.
- ROTHWELL, T.L.W., LOVE, R.J. AND EVANS, D.P. (1978) Studies on the role of histamine and 5-hydroxytryptamine in immunity against the nematode *Trichostrongylus colubriformis*. IV. Inhibition of the expulsion of worms transplanted into duodenum of immune guinea pigs. *International Archives of Allergy and Applied Immunology* 56, 457-464.
- ROTHWELL, T.L.W., WINDON, R.G., HORSBURGH, B.A. AND ANDERSON, B.H. (1993) Relationship between eosinophilia and responsiveness to infection with *Trichostrongylus colubriformis* in sheep. *International Journal for Parasitology* 23, 203-211.
- SALAWU, M.B., ACAMOVIC, T., STEWART, C.S. AND HOVELL, F.D.DeB. (1997) Quebracho tannins with or without Browse Plus (a commercial preparation of polyethylene glycol) in sheep diets: effect on digestibility of nutrients *in vivo* and degradation of grass hay *in sacco* and *in vitro*. *Animal Feed Science and Technology* 69, 67-78.
- SALAWU, M.B., ACAMOVIC, T., STEWART, C.S. AND HOVELL, F.D.DeB. (1999) Effects of feeding quebracho tannin diets, with or without a dietary modifier, on rumen function in sheep. *Animal Science* 69, 265-274.
- SANTIDRIAN, S. (1981) Intestinal absorption of D-glucose, D-galactose and L-leucine in male growing rats fed a raw field bean (*Vicia faba* L.) diet. *Journal of Animal Science* 53, 415-419.



- SCALBERT, A. (1991) Antimicrobial properties of tannins. *Phytochemistry* 30, 3875-3883.
- SCOFIELD, A.M. (1977) Intestinal absorption of hexoses in rats infected with *Nippostrongylus brasiliensis*. *International Journal for Parasitology* 7, 159-165.
- SCOFIELD, A.M. (1980) Effect of level of infection with *Nippostrongylus brasiliensis* on intestinal absorption of hexoses in rats. *International Journal for Parasitology* 10, 375-380.
- SELL, D.R., REED, W.M., CHRISMAN, G.L. AND ROGLER, J.C. (1985) Mucin excretion and morphology of the intestinal tract as influenced by sorghum tannins. *Nutrition Reports International* 31, 1369-1374.
- SELL, D.R., ROGLER, AND J.C. (1983) Effects of sorghum grain tannins and dietary protein on the activity of liver UDP-glucuronyltransferase. *Proceedings of the Society for Experimental Biology and Medicine* 174, 93-101.
- SIDDONS, R.C., PARADINE, J., BEEVER, D.E. AND CORNELL, P.R. (1985) Ytterbium acetate as a particulate-phase digesta-flow marker. *British Journal of Nutrition* 54, 509-519.
- SINGLETON, V.L. AND KRATZER, F.H. (1969) Toxicity and related physiological activity of phenolic substances of plant origin. *Journal of Agricultural and Food Chemistry* 17, 497-512.
- SMYTH, J.D. (1994) *Introduction to Animal Parasitology*. University Press, Cambridge.
- SPECIAN, R.D. AND OLIVER, M.G. (1991) Functional biology of intestinal goblet cells. *American Journal of Physiology* 260, C183-C193.
- STEEL, J.W., JONES, W.O. AND WAGLAND, B.M. (1990) The response of immune sheep to challenge with *Trichostrongylus colubriformis*: Enteric plasma loss and secretion of biogenic amines. *International Journal for Parasitology* 20, 1067-1073.
- STEEL, J.W., SYMONS, E.A. AND JONES, W.O. (1980) Effects of level of larval intake on the productivity and physiological and metabolic responses of lambs infected with *Trichostrongylus colubriformis*. *Australian Journal of Agricultural Research* 31, 821-838.
- STEVENSON, L.M., HUNTLEY, J.F., SMITH, W.D. AND JONES, D.G. (1994) Local eosinophil- and mast cell-related responses in abomasal nematode infections of lambs *FEMS Immunology and Medical Microbiology* 8, 167-174.
- STREIT, W. AND FENGAL, D. (1994) Purified tannins from *Quebracho colorado*. *Phytochemistry* 36, 481-484.



- SUTTLE, N.F., KNOX, D.P., ANGUS, K.W., JACKSON, F. AND COOP, R.L. (1992) Effects of dietary molybdenum on nematode and host during *Haemonchus contortus* infection in lambs. *Research in Veterinary Science* 52, 230-235.
- SYKES, A.R. AND COOP, R.L. (1976) Intake and utilisation of food by growing lambs with parasitic damage to the small intestine caused by daily dosing with *Trichostrongylus colubriformis* larvae. *The Journal of Agricultural Science, Cambridge* 86, 507-515.
- SYMONS, L.E.A. (1969) Pathology of gastrointestinal helminthiases. *International Review of Tropical Medicine* 3, 49-100.
- SYMONS, L.E.A. (1985) Anorexia: Occurrence, pathophysiology, and possible causes in parasitic infections. *Advance in Parasitology* 24, 103-133.
- TAYLOR, C.E. AND MURANT, A.F. (1966) Nematocidal activity of aqueous extracts from raspberry canes and roots. *Nematologica* 12, 488-494.
- TEBIB, K., BESANCON, P. AND ROUANET, J.-M. (1994) Dietary grape seed tannins affect lipoproteins, lipoprotein lipases and tissue lipid in rats fed hypercholestrolemic diets. *Journal of Nutrition* 124, 2451-2457.
- TEMBELY, S. (1998) Development and survival of infective larvae of nematode parasites of sheep on pasture in a cool tropical environment. *Veterinary Parasitology* 79, 81-87.
- TERRILL, T.H., DOUGLAS, G.B., FOOTE, A.G., PURCHAS, R.W., WILSON, G.F. AND BARRY, T.N. (1992a) Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum coronarium*) and perennial pasture. *The Journal of Agricultural Science, Cambridge* 119, 265-273.
- TERRILL, T.H., ROWAN, A.M., DOUGLAS, G.B. AND BARRY, T.N. (1992b) Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *Journal of the Science of Food and Agriculture* 58, 321-329.
- TOLERA, A., KJIAZAAL, K. AND ØRSKOV, E.R. (1997) Nutritive evaluation of some browse species. *Animal Feed Science Technology* 67, 181-195.
- UBER, C.L., ROTH, R.L. AND LEVY, D.A. (1980) Expulsion of *Nippostrongylus brasiliensis* by mice deficient in mast cells. *Nature* 287, 226-228.
- ULYATT, M.J. AND EGAN, (1979) Quantitative digestion of fresh herbage by sheep. V. The digestion of four herbages and prediction of sites of digestion. *The Journal of Agricultural Science, Cambridge* 92, 605-616.
- URQUIHART, G.M., ARMOUR, J., DUNCAN, J.L., DUNN, A.M. AND JENNINGS, F.W. (1987) *Veterinary Parasitology*. Longman Scientific & Technical, Bath Press, Avon.



- VALLET, J., ROUANET, J-M. AND BESANCO, P (1994) Dietary grape seed tannins: Effects on nutritional balance and on some enzymatic activities along the crypt villus axis of rat small intestine. *Annals of Nutrition and Metabolism* 38, 75-84.
- VAN SOEST, P.J. (1994) *Nutritional Ecology of the Ruminant*. 2nd Edition. Cornell University Press, London, UK.
- WAGHORN, G.C. (1990) Effect of condensed tannin on protein digestion and nutritive values of fresh herbage. *Proceedings of the Australian Society for Animal Production* 18, 412-415.
- WAGHORN, G.C., JOHN, A., JONES, W.T. AND SHELTON, I.D. (1987a) Nutritive values of *Lotus corniculatus* L. containing low and medium concentrations of condensed tannins for sheep. *Proceedings of the New Zealand Society of Animal Production* 47, 25-30.
- WAGHORN, G.C. AND JONES, W.T. (1989) Bloat in Cattle. 46. Potential of dock (*Rumex obtusifolius*) as an antibloating agent. *New Zealand Journal of Agricultural Research* 32, 227-235.
- WAGHORN, G.C. AND SHELTON, I.D. (1995) Effect of condensed tannins in *Lotus pedunculatus* on the nutritive value of ryegrass (*Lolium perenne*) fed to sheep. *The Journal of Agricultural Science, Cambridge* 125, 291-297.
- WAGHORN, G.C. AND SHELTON, I.D. (1997) Effect of condensed tannin in *Lotus corniculatus* on the nutritive value of pasture for sheep. *The Journal of Agricultural Science, Cambridge* 128, 365-372.
- WAGHORN, G.C., SHELTON, I.D. AND McNABB, W.C. (1994a) Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 1. Non-nitrogenous aspects. *The Journal of Agricultural Science, Cambridge* 123, 99-107.
- WAGHORN, G.C., SHELTON, I.D., McNABB, W.C. AND MCCUTCHEON, S.N. (1994b) Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 2. Nitrogenous aspects. *The Journal of Agricultural Science, Cambridge* 123, 109-119.
- WAGHORN, G.C., ULYATT, M.J., JOHN, A. AND FISHER, M.T. (1987b) The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *British Journal of Nutrition* 57, 115-126.
- WAGLAND, B.M., STEEL, J.W., WINDON, R.G. AND DINEEN, J.K. (1984) The response of lambs to vaccination and challenge with *Trichostrongylus colubriformis*: effect of plane of nutrition, and the inter-relationship between immunological responsiveness and resistance. *International Journal for Parasitology* 14, 39-44.
- WAKELIN, D (1978) Immunity to intestinal parasites. *Nature* 273, 617-620



- WAKELIN, D. (1994) Immunoparasitology. In: *Introduction to Animal Parasitology*, pp. 460-490 (Ed. J.D. Smyth) University Press, Cambridge.
- WAKELIN, D. (1996) Immunity to parasites: how parasitic infections are controlled. 2<sup>nd</sup> Edition. University Press, Cambridge.
- WALDO, D.R. (1973) Extent and partition of cereal grain starches digestion in ruminants. *Journal of Animal Science* 37, 1062-1074.
- WALL, R. AND STRONG, L. (1987) Environmental consequences of treating cattle with the antiparasitic drug ivermectin. *Nature* 327, 418.
- WALLACE, D.S., BAIRDEN, K., DUNCAN, J.L., ECKERSALL, D.S., FISHWICK, G., GILL, M., HOLMES, P.H., MCKELLAR, Q.Q., MURRAY, M., PARKINS, J.J. AND STEAR, M.J. (1998) The influence of dietary supplementation with urea on resilience and resistance to infection with *Haemonchus contortus*. *Parasitology* 116, 67-72.
- WALLER, P.J. (1997) Nematode parasite control of livestock in the tropics/subtropics: the need for novel approaches. *International Journal for Parasitology* 27, 1193-1201.
- WALLER, P.J. (1999) International approaches to the control of nematode parasites of livestock. *International Journal for Parasitology* 29, 155-164.
- WAN, J, M-F., HAW, M.P. AND BLACKBURN, G.L. (1989) Nutrition, immune function, and inflammation: an overview. *Proceedings of the Nutrition Society* 48, 315-335.
- WANG, Y., DOUGLAS, G.C., BARRY, T.N., FOOTE, A.G. AND PURCHAS, R.W. (1996a) Effect of condensed tannins upon the performance of lambs *Lotus corniculatus* and lucerne (*Medicago sativa*). *The Journal of Agricultural Science, Cambridge* 126, 87-98.
- WANG, Y., DOUGLAS, G.C., BARRY, T.N. AND FOOTE, A.G. (1996b) Effect of condensed tannins in *Lotus corniculatus* upon the digestion of methionine and cysteine in the small intestine of sheep. *The Journal of Agricultural Science, Cambridge* 127, 413-421.
- WANG, Y., WAGHORN, G.C., DOUGLAS, G.C., WILSON, G.F. AND BARRY, T.N. (1994) The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *British Journal of Nutrition* 57, 219-222.
- WELLS, P.D. (1963) Mucin-secreting cells in rats infected with *Nippostrongylus brasiliensis*. *Experimental Parasitology* 14, 15-22.
- WEIR, D.M. AND STEWART, J. (1993) *Immunology*. 7th Edition. Churchill Livingston, London.



- WHEATER, P.R., BRUKITT, H.G. AND DANIELS, V.G. (1987) *Functional Histology: A text and colour atlas* Churchill Livingstone, UK.
- WHITFIELD, P.J. (1993) Parasitic helminths. In: *Modern Parasitology. A textbook of parasitology*. pp. 24-52 (Ed. F.E.G. Cox) 2nd Edition, Blackwell Scientific Publications Inc., Oxford.
- WILSON, W.D. AND FIELD, A.C. (1983) Absorption and secretion of calcium and phosphorus in the alimentary tract of lambs infected with daily doses of *Trichostrongylus colubriformis* and *Ostertagia circumcincta* larvae. *Journal of Comparative Pathology* 93, 61-71.
- WINDON, R.G. AND DINEEN, J.K. (1981) The effect of selection of both sire and dam on the response of F<sub>1</sub> generation lambs to vaccination with irradiated *Trichostrongylus colubriformis* larvae. *International Journal for Parasitology* 11, 11-18.
- WINER, B.J., BROWN, D.R. AND MICHELS, K.M. (1991) *Statistical Principles in Experimental Design*, 3<sup>rd</sup> Edition. McGraw-Hill Inc., New York.
- WINTER, M.D., WRIGHT, C. AND LEE, D.L. (1997) The mast cell and eosinophil response of young lambs to a primary infection with *Nematodirus battus*. *Parasitology* 114, 189-193.
- WOOLASTON, R.R. AND PIPER, L.R. (1996) Selection of Merino sheep for resistance to *Haemonchus contortus*: genetic variation. *Animal Science* 62, 451-460.
- YU, F., MCNABB, W.C., BARRY, T.N. AND MOUGHAN, P.J. (1996a) Effect of heat treatment upon the chemical composition of cottonseed meal and upon the reactivity of cottonseed condensed tannins. *Journal of the Science of Food and Agriculture* 72, 263-272.
- YU, F., MCNABB, W.C., BARRY, T.N. AND WAGHORN, G.C. (1995) Effect of condensed tannin in cottonseed hulls upon the *in vitro* degradation of cottonseed kernel proteins by rumen micro-organisms. *Journal of the Science of Food and Agriculture* 69, 223-234.
- YU, F., MOUGHAN, P.J. AND BARRY, T.N. (1996b) The effect of condensed tannins from heated and unheated cottonseed on the ileal digestibility of amino acids for the growing rat and pig. *British Journal of Nutrition* 76, 359-371.

## APPENDIX

### SOLUTIONS AND BUFFERS

#### Phosphate Buffered Saline (PBS) (section 2.3.1)

|                                  |        |
|----------------------------------|--------|
| NaCl                             | 8.00 g |
| KCl                              | 0.20 g |
| Na <sub>2</sub> HPO <sub>4</sub> | 1.15 g |
| KH <sub>2</sub> PO <sub>4</sub>  | 0.20 g |

Dissolve in sequence making the final volume to 1 litre with distilled water and adjust the pH to 7.2, if necessary.

#### Hanks' Balanced Salt Solution (HBSS) (section 2.3.3.1; 2.3.5)

##### *Solution 1*

|                                       |         |
|---------------------------------------|---------|
| NaCl                                  | 168.0 g |
| KCl                                   | 8.0 g   |
| KH <sub>2</sub> PO <sub>4</sub>       | 2.0 g   |
| NaHPO <sub>4</sub> .2H <sub>2</sub> O | 2.0 g   |
| 0.2 % (w/v) Phenol Red                | 200ml   |

##### *Solution 2*

|                                      |       |
|--------------------------------------|-------|
| CaCl <sub>2</sub> .2H <sub>2</sub> O | 3.7 g |
| MgCl <sub>2</sub> .6H <sub>2</sub> O | 2.0 g |



Both solution 1 and 2 are made to a volume of 2 litres with distilled water. Mix 110ml solution 1 and 110ml solution 2 and make to a volume of 1 litre with distilled water. The final solution is pH adjusted to 7.2 with M NaOH.

#### **0.2 % (w/v) Phenol Red**

Phenol red                      2 g

1.75 M NaOH                100 ml

The volume is made up to 950 ml with distilled water and pH adjusted to 7.0 with 1.75 M NaOH. The final volume of 1 litre is made up with distilled water.

#### **10 % (v/v) Neutral Buffered Formalin (section 2.3.3.1)**

Formaldehyde [40 % solution]            100 ml

NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O                                4.0 g

Na<sub>2</sub>HPO<sub>4</sub>                                        6.5 g

The volume is made up to 1 litre with distilled water.

#### **Helminthological Iodine (section 2.3.3.2)**

Potassium iodide            907 g

Dissolve in 650 ml boiling water.

Iodine                                510 g

The final volume is made up to 1 litre with distilled water.

### **Carpentiers Eosinophil Counting Solution (section 2.4.2)**

2% (w/v) aqueous Eosin Y 2.0 ml

40% Formaldehyde saturated with  $\text{CaCO}_3$  3.0 ml

The final volume is made up to 100 ml with distilled water. Solution is prepared fresh each week.

### **Biuret Reagent (section 2.4.4)**

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  3.0 g

Dissolve in approximately 500 ml distilled water.

Sodium potassium tartrate [ $\text{KOO}-(\text{CHOH})_2-\text{COONa} \cdot 4\text{H}_2\text{O}$ ] 9.0 g

Potassium iodide 5.0 g

When the solution is clear, add 100 ml NaOH (6 mol/l) and dilute to 1 litre with distilled water.

### **Biuret Blank Reagent (section 2.4.4)**

Prepared exactly as the biuret reagent but no  $\text{CuSO}_4$  added.

### **Bromocresol Green (BCG) Reagent (section 2.4.5)**

BCG (3,3',5,5'-tetrabromo-*m*-cresolsulfonphalein) 105 g

Succinic acid 8.85 g

Sodium azide 100 mg

Brij-35 (300 g/l) 4 ml

Dissolve in approximately 950 ml distilled water. Adjust the pH of the solution to 4.15 to 4.25 with NaOH (6 mol/l) and make volume to 1 litre with distilled water. Store at room temperature in a tightly closed polyethylene bottle.



**Carbonate/Bicarbonate Buffer (section 2.5.1)**

|                                 |        |
|---------------------------------|--------|
| Na <sub>2</sub> CO <sub>3</sub> | 1.59 g |
| NaHCO <sub>3</sub>              | 2.93 g |
| NaN <sub>3</sub>                | 0.20 g |

Dissolve in 300 ml distilled water and adjust pH to 9.6. Final volume is made up to 1 litre with distilled water.

**PBS-Tween (section 2.5.1)**

|          |         |
|----------|---------|
| PBS      | 1000 ml |
| Tween 20 | 0.5 ml  |

**Digestion Acid (section 2.7)**

|                                |        |
|--------------------------------|--------|
| Orthophosphoric acid (880 g/l) | 250 ml |
| Manganese sulphate (100 g/l)   | 50 ml  |
| Sulphuric acid                 | 250 ml |

Dilute to give final volume of 1 litre with distilled water.

**Composition of Spec Lamb (g/kg) (section 4.2.1)**

|                                |     |
|--------------------------------|-----|
| Barley                         | 465 |
| Oatfeed                        | 160 |
| Grassmeal                      | 195 |
| Fishmeal                       | 55  |
| Minerals/Vitamins <sup>1</sup> | 25  |
| Nutrimol                       | 100 |